Caracterización fenotípica y funcional del monocito como célula implicada en el estado de inflamación sistémica persistente en ancianos y en pacientes infectados por VIH-1 como modelo de envejecimiento prematuro

Tesis Doctoral

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UNIVERSIDAD DE SEVILLA

Facultad de Medicina

Departamento de Medicina

Doctorado en Biología Molecular y Biomedicina

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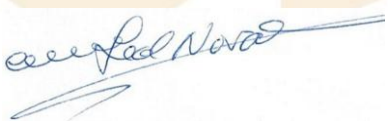
CERTIFICAN:

Que el trabajo titulado "Caracterización fenotípica y funcional del monocito como célula implicada en el estado de inflamación sistémica en ancianos y en pacientes infectados por VIH-1 como modelo de envejecimiento prematuro", presentado por la Licenciada en Veterinaria **Dña. REBECA DE PABLO BERNAL,** ha sido realizado bajo nuestra dirección y asesoramiento en el Laboratorio de Inmunovirología del Instituto de Biomedicina de Sevilla (IBIS).

Concluido el trabajo experimental y bibliográfico, autorizamos la presentación y la defensa de esta Tesis Doctoral, para que sea juzgada por el tribunal correspondiente.

Sevilla, a 14 de Octubre de 2016.



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Introducción

El desarrollo y evolución en la terapia antirretroviral combinada (cART), por suprimir la replicación viral hasta niveles sanguíneos de Carga Viral (CV) indetectables, ha supuesto un hito en la historia de la infección por el Virus de la Inmunodeficiencia Humana tipo 1 (VIH-1), el acceso al cART en los países desarrollados ha favorecido la reducción de la morbilidad y mortalidad asociada a SIDA (1,2). Sin embargo, esta situación más favorable ha hecho emerger otra realidad clínica, caracterizada por una mayor prevalencia de enfermedades no SIDA (ENOS) (3). En este sentido, ha sido descrito por ciertos autores una mayor prevalencia y precocidad en el desarrollo de enfermedades como cáncer, cardiovascular, ósea, renal, diabetes y transtornos neurocognitivos (4,5) (también denominadas como enfermedades asociadas a la edad) en pacientes infectados por VIH bajo cART que en la población general de similar edad. Esto es debido, a que el cART podría no restaurar completamente el daño inmunológico que se produce en la infección por el VIH (6). En este sentido, incluso algunos autores han señalado que ciertos fármacos antirretrovirales podrían inducir efectos proinflamatorios y aumento de la activación celular, estas observaciones han sido descritas a partir de cultivos de células humanas *in-vitro* y modelos murinos *in-vivo* (7, 8), sugiriendo un papel activo y deletéreo sobre el sistema inmune de los pacientes infectados por VIH bajo ciertos cART.

Por analogía con el envejecimiento natural, definido como la disminución progresiva de la respuesta inmunitaria que afecta a todos los componentes del sistema inmunológico tanto innato como adaptativo (9), lo que contribuye de manera importante a la morbilidad y mortalidad de los ancianos, ya que favorece una mayor incidencia y reactivación de enfermedades infecciosas (10), así como el desarrollo de patologías autoinmunes y cáncer (11-17). Ha sido sugerido por varios autores que el paciente infectado por VIH posee un proceso de envejecimiento prematuro y acentuado del proceso natural de senescencia (4, 18). Esto es debido a que el paciente infectado por VIH naïve y los ancianos no infectados por el virus a menudo presentan similares rasgos de desgaste inmunológico como reactivaciones de infecciones silentes como citomegalovirus (CMV), reducción de la función del timo, disminución del número de células naïve y expansión del número de células T CD57+, entre otros (19-28) (resumidos en Tabla 1), que además se asocian a la aparición de enfermedad en ambos escenarios (29).

Tabla 1. Posibles similitudes entre los cambios inmunológicos asociados a la edad y a la infección por VIH.

Parámetro	VIH- ≥65años	VIH+ naïve	VIH+ cART
Aumento activación céls.T	Controvertido	SÍ	Posible
Expansión céls.T CD57+	SÍ	SÍ	Sí
Expansión clones específicos CMV	SÍ	SÍ	SÍ
Aumento títulos IgG anti-CMV	SÍ	SÍ	SÍ
Disminución ratio céls. naïve/memoria	SÍ	SÍ	Posible
Bajo potencial proliferativo céls. T	SÍ	SÍ	Posible
Reducida respuesta a vacunas	SÍ	SÍ	Posible
Reducción función del timo	SÍ	SÍ	Desconocido
Aumento niveles plasmáticos IL6	SÍ	SÍ	Desconocido

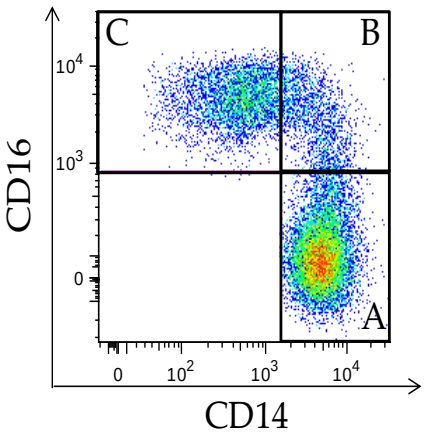
Sin embargo, si el tratamiento antirretroviral a largo plazo restaura esas alteraciones está siendo estudiado, siendo los defectos en el compartimento celular T, los mejores descritos hasta la actualidad. Si el tratamiento a largo plazo normaliza o reduce la alteración en marcadores inflamatorios solubles de pacientes infectados por VIH y en qué medida con respecto a poblaciones no infectadas de diferentes edades, es todavía incierto. Es por este motivo, que el primer objetivo de la presente tesis doctoral, fue comparar los niveles de mediadores de inflamación solubles en pacientes infectados por VIH naïves al tratamiento antirretroviral y bajo cART a largo plazo con dos poblaciones de referencia no infectadas por VIH de diferentes edades: 1) jóvenes, apareados por edad a la población infectada por VIH y 2) ancianos. Este objetivo fue analizado en el trabajo: "**TNF-α levels in HIV-infected patients after long-term suppressive cART persist as high as in elderly, HIV-uninfected subjects."** (J Antimicrob Chemother. 2014).

Los mecanismos implicados en el estado inflamatorio crónico de bajo grado observado tanto en pacientes como ancianos son desconocidos. Nosotros pensamos que una de las causas que podría asociarse a los altos niveles de marcadores proinflamatorios observados en ambos escenarios, podría ser la elevada activación de monocitos circulantes. Los monocitos, son esencialmente células efectoras y mediadoras de la respuesta inflamatoria (30,31). Los monocitos son fagocitos que derivan de la médula ósea, poseen vida corta (1-3 días en circulación sanguínea) y están implicados en la respuesta inmune innata ante patógenos (32-35). En humanos, recientemente se han descrito tres subpoblaciones de monocitos dependiendo de la expresión de dos de sus receptores de superficie el CD14 (receptor del complejo de lipopolisacárido y de la proteína de unión al lipopolisacárido o LBP) y CD16. (FcγRIII). Así se identifica una subpoblación de monocitos clásicos (CD14++CD16−), los intermedios (CD14++CD16+) y los no clásicos (CD14dimCD16+) (36). Uno de los rasgos a destacar entre las tres subpoblaciones es que presentan heterogeneidad: en tamaño, complejidad, receptores (de membrana e intracelulares). De forma interesante, en cuanto a la funcionalidad celular también se han descrito diferencias (37-39). La identificación de las tres subpoblaciones es relativamente reciente (2010) y actualmente su caracterización está siendo objeto de estudios básicos. A continuación se resumen las principales características de las tres subpoblaciones:

A. Monocitos clásicos (CD14++CD16−): subpoblación de mayor tamaño y complejidad de núcleo, mayor capacidad de fagocitosis y productora de ROS in-vitro. Mayor respuesta frente a agonistas de TLR-2 y 4.

B. Monocitos intermedios (CD14++CD16+): características intermedias entre las tres subpoblaciones en cuanto a tamaño y complejidad, mayor expresión en membrana de MHCII.

C. Monocitos no clásicos (CD14dimCD16+): subpoblación más sencilla y pequeña**.** Respuesta ante agonistas endosómicos TLR 7 y 8. Implicada en el rodamiento y adhesión al endotelio vascular



Algunos autores, han descrito alteraciones en marcadores de activación, así cómo en el número y distribución de las diferentes subpoblaciones de monocitos en el envejecimiento natural. Existen asociaciones entre el número de monocitos no clásicos, la disminución de la expresión de la selectina CD62L (40) y la disminución de la capacidad fagocítica con la edad avanzada (41). A pesar, de la relevancia de estas células como potencial mecanismo implicado en el estado de inflamación persistente que ha sido descrito en ancianos, los cambios fenotípicos, y funcionales y su relación con parámetros inflamatorios solubles y otros factores que podrían estar asociados como son, la infección latente por CMV y la enfermedad neurocognitiva en ancianos son desconocidos.

El siguiente objetivo de la presente tesis doctoral, fue analizar los cambios fenotípicos y funcionales en las tres subpoblaciones de monocitos con la edad. Así como analizar su asociación con el estado de inflamación persistente observado en ancianos, y su relación con la infección latente por CMV y con el deterioro neurocognitivo.

Este objetivo fue abordado en el trabajo: **"Monocyte phenotype and polyfunctionality are associated with elevated soluble inflammatory markers, cytomegalovirus infection, and functional and cognitive decline in elderly adults**." (J Gerontol A Biol Sci Med Sci 2015, en prensa).

Recientemente en el escenario de la infección por VIH han aparecido trabajos que describen la elevada activación de monocitos (42,43) y marcadores solubles asociados a estas células (44-46). Estas alteraciones, que se ha visto que incluso persisten en pacientes infectados por VIH bajo cART a largo plazo, además se han asociado con el desarrollo de ENOS en estos pacientes (47-49). En este sentido, la cuestión que se deriva de estas observaciones es si los mecanismos asociados a la alteración inflamatoria en pacientes bajo cART y en el envejecimiento natural son similares y en qué medida comparten rasgos. Recientemente, algunos autores han sugerido que los monocitos de pacientes presentan alteraciones compatibles con un envejecimiento prematuro de estas células (50, 51). Sin embargo, todavía la funcionalidad (en cuanto a la capacidad de producción de citoquinas *ex-vivo* e *in-vitro*) simple y compuesta por más de una citoquina de forma simultánea (polifuncionalidad) de los monocitos de pacientes bajo cART es desconocida. Por esto, el siguiente objetivo de esta tesis doctoral fue, caracterizar de forma detallada los cambios fenotípicos y funcionales de los monocitos de pacientes infectados por VIH bajo cART, incluyendo las tres subpoblaciones de monocitos, tanto *ex-vivo* como *in-vitro* y compararlo con los de una población no infectada por VIH envejecida. Este objetivo fue analizado en la publicación "**Phenotype and polyfunctional deregulation involving IL-6- and IL-10-producing monocytes of HIV-infected patients on cART differ from those of aging"** (The Journal of Infectious Diseases 2015, en prensa).

En la Figura 1, se resumen los efectos de la infección por VIH-1 y su tratamiento en inflamación e inmunosenescencia.

Las hipótesis y objetivos de la presente tesis doctoral fueron:

Hipótesis 1: el estado inflamatorio crónico de pacientes infectados por VIH-1 comparte alteraciones con el observado en envejecimiento natural. Presentando, sin embargo, rasgos propios que hacen que no sea compatible con el proceso natural de envejecimiento prematuro de su sistema inflamatorio.

> **Objetivo 1:** Comparar los niveles de mediadores de inflamación solubles en pacientes infectados por VIH naïves al tratamiento y bajo cART a largo plazo con dos poblaciones de referencia no infectadas por VIH de diferentes edades: jóvenes y ancianos. Este objetivo fue analizado en el trabajo: "**TNF-α levels in HIV-infected patients after long-term suppressive cART persist as high as in elderly, HIV-uninfected subjects."** (J Antimicrob Chemother. 2014).

Hipótesis 2: la activación y alteración en la función de monocitos con avanzada edad se asocia con el estado de inflamación crónica persistente observado en ancianos y con otros factores como la infección latente por CMV y deterioro neurocognitivo.

> **Objetivo 2:** analizar los cambios fenotípicos y funcionales en las tres subpoblaciones de monocitos con la edad. Así como analizar su asociación con el estado de inflamación persistente observado en ancianos, y su relación con la infección latente por CMV y con el deterioro neurocognitivo.
> Este objetivo fue abordado en el trabajo: **"Monocyte phenotype and polyfunctionality are associated with elevated soluble inflammatory markers, cytomegalovirus infection, and functional and cognitive decline in elderly adults**." (J Gerontol A Biol Sci Med Sci 2015, en prensa).

Hipótesis 3: los monocitos de pacientes infectados por VIH bajo cART presentan alteraciones fenotípicas y funcionales diferentes a las observadas en el envejecimiento natural.

> **Objetivo 3:** caracterizar de forma detallada los cambios fenotípicos y funcionales de los monocitos de pacientes infectados por VIH bajo cART, incluyendo las tres subpoblaciones de monocitos, tanto *ex-vivo* como *in-vitro* y compararlo con los de una población envejecida. Este objetivo fue analizado en la publicación "**Phenotype and polyfunctional deregulation involving IL-6- and IL-10-producing monocytes of HIV-infected patients on cART differ from those of aging"** (The Journal of Infectious Diseases 2015, ,en prensa).

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TNF-α levels in HIV-infected patients after long-term suppressive cART persist as high as in elderly, HIV-uninfected subjects

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Background: Chronic and systemic inflammatory alterations occur in HIV-infected patients and elderly uninfected subjects and in both scenarios these alterations are associated with the development of chronic morbidities and mortality. However, whether the levels of inflammatory alterations in untreated HIV-infected patients and elderly individuals are similar is unknown. Moreover, whether long-term antiretroviral therapy normalizes inflammatory alterations compared with HIV-uninfected persons of different age is not known.

Methods: We analysed soluble inflammatory levels [high-sensitivity C-reactive protein, interferon (IFN)-γ, tumour necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-8 and IL-17] in a cohort of viraemic HIV-infected patients compared with (i) age-matched, (ii) elderly and (iii) non-survivor elderly, uninfected healthy controls. We longitudinally analysed the effect of long-term 48 and 96 week suppressive combined antiretroviral therapy (cART) on the soluble inflammatory levels compared with those found in control subjects.

Results: Baseline IL-6 and IL-8 levels were at similar or lower concentrations in untreated patients compared with healthy elderly individuals. However, TNF-α and IFN-γ levels broadly exceeded those found in survivors and non-survivor elderly individuals. Long-term suppressive cART normalized most of the inflammatory markers, with the exception of TNF-α levels, which persisted as high as those in elderly non-survivor controls.

Conclusions: Chronic inflammatory alterations associated with HIV infection are maintained at a different level from those of ageing. The persistent alteration of TNF-α levels in HIV-infected patients might cause tissue damage and have implications for developing non-AIDS-defining illnesses, even when HIV replication is long-term controlled by cART.

Keywords: HIV infection, inflammation, ageing

Introduction

Ageing and HIV infection are associated with profound changes in the immune system, with marked similarities. Both induce several defects that are particularly associated with T cell function, such as thymic involution, which reduces the numbers of circulating naive T cells.[1–3] Although less described, persistent inflammation and a hypercoagulable altered state have been reported for HIV infection and ageing.[4] Untreated HIV infection is associated with persistently high plasma interleukin (IL)-6 and tumour necrosis factor (TNF)-α concentrations.[5] Additionally, chronic elevation of plasma inflammatory markers has been described in HIV-uninfected elderly subjects.[6] Furthermore, these alterations are strongly associated with the development of morbidity and mortality in the elderly and in those with HIV infection.[7–9] However, no previous studies have compared the inflammatory alterations of elderly individuals with those of naive, HIV-infected patients to address whether these alterations are similar and/or occur at the same magnitude for antiretroviral treatment.

Once patients are treated with combined antiretroviral therapy (cART), the suppression of HIV replication results in a substantial decrease in AIDS-related morbidity and mortality,[10] resulting in an increased life expectancy[11] and a more aged HIV-infected population. However, cART mediates an immune restoration that, unfortunately, is incomplete even in long-term virologically suppressed patients.[12] Thus, effectively treated patients show an immune dysfunction that includes inflammatory alterations,[13] which translates to higher risk for developing chronic diseases (termed non-AIDS morbidities) when compared with similar-aged, HIV-uninfected persons.[14] Cross-sectional and longitudinal studies have shown that short-term cART reduces but does not normalize systemic inflammatory alterations.[5] However, whether long-term antiretroviral therapy normalizes or reduces these alterations compared with HIV-uninfected persons of different age is not known.

Thus, the aim of this study was to compare the levels of inflammatory markers in HIV-infected persons immediately before and after 48 and 96 weeks of long-term suppressive treatment, and also compare them with the inflammatory states of age-matched, elderly HIV-uninfected controls.

Methods

Study participants

Naive-for-cART HIV-infected patients were retrospectively selected from the Infectious Disease Unit at Virgen del Rocío University Hospital in Seville (Spain). Thirty-nine asymptomatic and consecutive Caucasian patients were included according to sample availability from the HIV BioBank of the Spanish AIDS Research Network. Individuals were analysed at baseline ($n=39$, HIV_0) and after 48 ($n=39$, HIV_{48}) and 96 weeks ($n=19$, HIV_{96}) of suppressive cART (defined as persistent undetectable viral load).

HIV-infected patients were compared with age-matched, HIV-uninfected healthy subjects ['Young' (Y) group, $n=26$] and with elderly subjects belonging to the previously described CARRERITAS cohort.[15] Briefly, free-living volunteers and nursing home residents from Seville (Spain) were asked to participate in this cohort. Inclusion criteria included subjects aged ≥65 years and self-sufficient health status. Exclusion criteria included diagnosis of dementia or any of the following situations during the last 6 months: (i) clinical data of active infections; (ii) hospital admission; (iii) antitumour therapy; or (iv) any treatment that could influence their immune status (mainly corticosteroids). Demographic data and blood samples were obtained at baseline (2008) and at the end of follow-up (2010), when all participants were contacted again to assess survival rates. For this study, consecutive baseline (at the cohort inclusion) samples from the CARRERITAS cohort were selected for two elderly control groups according to sample availability: individuals >65 years who survived through the 2 year follow-up of this cohort were selected as the 'Elderly Survivor' group ($n=26$). Individuals >65 years old who died during the follow-up despite being healthy at baseline were selected as the 'Elderly Non-survivor' group ($n=26$).

All necessary institutional or ethical review board approvals were obtained and written informed consent was obtained from all study participants.

Laboratory methods

Absolute CD4 T cell counts (cells/mm^3) were determined using an Epics XL-MCL flow cytometer (Beckman-Coulter, Brea, CA, USA). The plasma HIV-1 RNA concentration (HIV-RNA copies/mL) was measured using quantitative PCR (COBAS Ampliprep/COBAS Taqman HIV-1 test, Roche Molecular Systems, Basel, Switzerland) according to the manufacturer's protocol. The detection limit for this assay was 20 HIV-RNA copies/mL. Plasma samples were tested for a hepatitis B virus (HBV)-related marker [hepatitis B surface antigen (HBsAg)] using an HBV ELISA (Siemens Healthcare Diagnosis, Malvern, PA, USA). Hepatitis C virus (HCV) RNA was detected using a commercially available PCR procedure (COBAS Amplicor, Roche Diagnosis, Barcelona, Spain) with a detection limit of 15 IU/mL. HCV exposure (anti-HCV) was detected using an HCV ELISA (Siemens Healthcare Diagnosis).

Cytomegalovirus (CMV) serostatus

Serum samples were screened for CMV IgG antibodies using enzyme immunoassay test kits (GenWay, San Diego, CA, USA) according to the manufacturer's instructions.

Measurement of inflammatory markers

Soluble plasma concentrations of the inflammatory markers interferon (IFN)-γ, IL-1β, IL-6, IL-8, IL-17 and TNF-α were determined in duplicate and included variability interplate controls (<20% variability was considered) using a two-site sandwich ELISA technique (R&D, San Diego, CA, USA). Plates were read using the ImageQuant LAS 4000 Mini imaging system (GE Healthcare Bio-Sciences, Uppsala, Sweden). The results were analysed using Q-view software (Quansys Bioscience, Logan, UT, USA) by automatically choosing the best curve-fitting regression models. High-sensitivity C-reactive protein (hsCRP) levels were determined through an immunoturbidimetric assay of sera using COBAS 701® (Roche Diagnostics, Mannheim, Germany).

Statistical analysis

All continuous variables are expressed as median (IQR). Categorical variables are expressed as *n* (%). Differences between groups were analysed using the χ^2 test for categorical variables and the Mann–Whitney *U*-test, Friedman test and Wilcoxon test were used for unmatched and matched continuous variables. *P* values <0.05 were considered significant. Statistical analysis was performed using SPSS version 20 (SPSS, Chicago, IL, USA) and Prism version 5.0 (GraphPad Software) was used to generate graphs.

Results

Characteristics of the study subjects

The demographic and immunovirological characteristics of all study participants are shown in Table 1. CMV serostatus was highly prevalent in all study groups and was 92%, 81%, 92% and 96% in HIV-infected patients, young, elderly and elderly non-survivor groups, respectively. There were no statistical differences in CMV status among the different study groups. The median CD4 T cell count was 350 cells/mm^3 for HIV-infected patients at baseline and 9 of 39 (23%) had CD4 T cell counts <200 cells/mm^3, despite a median time of diagnosis of only 3.2 months. After 48 weeks of cART, CD4 T cell counts reached a median of 583 cells/mm^3 and 3 of 39 (8%) had active HCV infection. All study participants were negative for HBsAg. The median $\log_{10}$ HIV RNA copies/mL was 4.5 at baseline and HIV suppression was achieved for all patients at a maximum of 48 weeks of therapy.

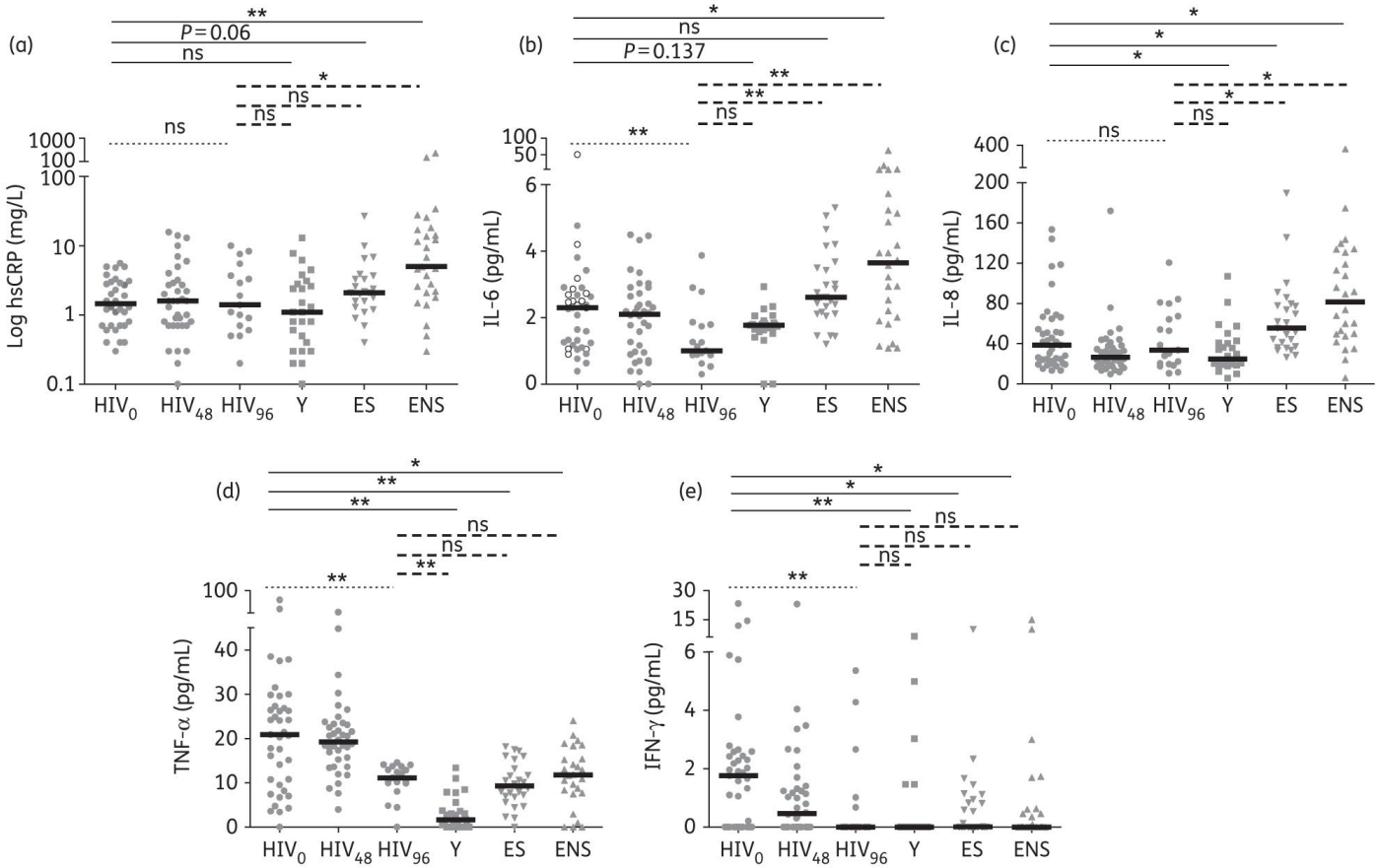

Figure 1. Plasma concentrations of inflammatory markers in naive HIV-infected patients immediately before cART (HIV_0) and after 48 and 96 weeks since cART initiation (HIV_{48} and HIV_{96}, respectively) and in different HIV-uninfected controls: young (Y), elderly survivor (ES) and elderly non-survivor (ENS) groups. (a) hsCRP, (b) IL-6, (c) IL-8, (d) TNF-α and (e) IFN-γ concentrations. Open circles represent patients >1 year since HIV diagnosis. The Friedman test (dotted line) was used to analyse differences during the follow-up period. The Mann–Whitney *U*-test (continuous lines) was used to compare the level of inflammatory markers between HIV-infected subjects at baseline and different age groups of healthy subjects. The Mann–Whitney *U*-test (dashed lines) was used to compare the levels of inflammatory markers between HIV-infected subjects at 96 weeks of suppressive cART and different age control groups. *$P<0.05$ and **$P<0.001$. ns, not significant.

inhibitor (NRTI)-containing cART versus an NRTI-sparing regimen [$n=23$ (59%) versus $n=16$ (41%), respectively] (data not shown).

Discussion

The results presented herein showed a heterogeneous and different level of chronic inflammatory alteration between HIV infection and ageing.

In accordance with other authors,[16] our results demonstrate the profound disruption that HIV causes in the inflammatory state of untreated patients when compared with similar-aged controls. In addition, this is the first study to show the different magnitudes of systemic inflammatory alterations between ageing and HIV infection. However, these results must be put into the context of the present cohort (reduced sample size, 23% of the patients had CD4 T cell counts <200 cells/mm^3, the median nadir CD4 T cell was 293 cells/mm^3, 8% of patients had a CDC-C event and short time since diagnosis of HIV); therefore, caution should be used when generalizing the results and making comparisons with previously published reports.

Chronic immune activation and systemic inflammation are likely to be the major causes of systemic ageing of physiological functions and the driving mechanisms for developing chronic diseases, termed age-related diseases, in HIV-uninfected elderly subjects. These observations have also been described for HIV[17] and have led to growing concern that HIV-infected persons suffer from accelerated or premature immunosenescence and ageing of the inflammatory system.[4,18] Persistent viral infections such as CMV or HIV that stimulate and exhaust the immune system may therefore play a major role in driving the ageing of the immune system. Our cohort presents high CMV seroprevalence in agreement with other Spanish cohorts, estimated to be 93.0% in adulthood.[15,19] Thus, the results presented herein suggest that although untreated HIV-infected patients and elderly uninfected subjects presented an altered chronic inflammatory state, HIV infection and not CMV coinfection might be the major cause favouring the inflammatory differences that we described for TNF-α and IFN-γ, which broadly exceed those of ageing. In this sense, we cannot exclude the possibility that the high hsCRP and IL-6 levels seen in elderly groups could be attributable to CMV reactivations and new

reinfections that occur more frequently in older people than in the young.[20]

Interestingly, we noticed that these alterations occurred shortly after infection (the median time since HIV diagnosis in the cohort was 3.2 months). Remarkably, we noticed that these alterations seemed to be time dependent. In this regard, only naive patients with >1 year since diagnosis showed significantly higher IL-6 levels compared with age-matched controls. Accordingly, higher IL-6 levels have been reported in naive patients compared with controls in data from a Strategies for Management of Antiretroviral Therapy study,[5] but this result is in contrast to other studies.[21] Therefore, our results suggest that these discordant results could be attributable to differing times since HIV diagnosis in patients from different cohorts. Consequently, these results showed the precocity of the inflammatory alterations and support the onset of cART as soon as possible.

Antiretroviral treatment tended to reduce systemic inflammation. However, only IL-8 levels were normalized after the first year of therapy. In contrast, 2 years of suppressive cART (≥1 year of virological suppression) were needed to normalize IFN-γ and IL-6 levels. However, the TNF-α level remained elevated, despite 96 weeks of suppressive cART. Although we cannot exclude the possibility that the TNF-α level could normalize after a longer period of treatment, one possible explanation for this heterogeneous profile could be that TNF-α is secreted by different cell types but mainly by monocytes upon TLR4 lipopolysaccharide (LPS)-mediated activation. Furthermore, it is known that low LPS concentrations cause the activation and release of high amounts of TNF-α by these cells *in vitro*[22] and we recently reported that long-term cART did not normalize levels of sCD14, which is another monocyte activation marker.[23] Thus, these activated monocytes might be the major source of TNF-α. Persistent inflammatory and hypercoagulable states that remain altered, even when HIV replication has been well controlled by cART for a long time, can damage several tissues, which leads to cumulative harm that might end in chronic diseases or non-AIDS illnesses in HIV-infected patients. In this sense, the TNF-α concentration predicts cardiovascular risk and mortality in non-HIV-infected elderly subjects.[8,24] Hence, these results support approaches involving strategies to reduce systemic TNF-α levels and may be important for reducing cardiovascular disease rates. Our results extend knowledge of inflammatory alterations after 96 weeks of suppressive treatment and provide further evidence of incomplete immune restoration mediated by cART even in long-term virologically suppressed patients.[12,23] The potential mechanisms driving persistent inflammation could be: (i) microbial translocation caused by barrier defects in gut-associated lymphoid tissue related to HIV pathogenesis; (ii) residual HIV proteins in virologically suppressed patients that could directly activate the innate immune system; (iii) reactivation of endogenous pathogens such as *Mycobacterium tuberculosis* and herpesviruses; or (iv) dysfunctional immunoregulatory factors.

In summary, our results demonstrate that the inflammatory alterations associated with chronic HIV infection are maintained at a different level from those during ageing. The persistent alteration of inflammatory markers, even after long-term suppressive cART, make it necessary to focus further investigations on the mechanisms that are the sources of this inflammation and potential therapeutic targets to reduce non-AIDS-defining illnesses and restore health to effectively treated patients.

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Transparency declarations

None to declare.

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Original Article

Monocyte Phenotype and Polyfunctionality Are Associated With Elevated Soluble Inflammatory Markers, Cytomegalovirus Infection, and Functional and Cognitive Decline in Elderly Adults

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Abstract

Monocytes are mediators of the inflammatory response and include three subsets: classical, intermediate, and nonclassical. Little is known about the phenotypical and functional age-related changes in monocytes and their association with soluble inflammatory biomarkers, cytomegalovirus infection, and functional and mental decline. We assayed the activation ex vivo and the responsiveness to TLR2 and TLR4 agonists in vitro in the three subsets and assessed the intracellular production of IL1-alpha (α), IL1-beta (β), IL-6, IL-8, TNF-α, and IL-10 of elderly adults (median 83 [67–90] years old; $n = 20$) compared with young controls (median 35 [27–40] years old; $n = 20$). Ex vivo, the elderly adults showed a higher percentage of classical monocytes that

expressed intracellular IL1-α ($p = .001$), IL1-β ($p = .001$), IL-6 ($p = .002$), and IL-8 ($p = .007$). Similar results were obtained both for the intermediate and nonclassical subsets and in vitro. Polyfunctionality was higher in the elderly adults. The functionality ex vivo was strongly associated with soluble inflammatory markers. The activation phenotype was independently associated with the anti-cytomegalovirus IgG levels and with functional and cognitive decline. These data demonstrate that monocytes are key cell candidates for the source of the high soluble inflammatory levels. Our findings suggest that cytomegalovirus infection might be a driving force in the activation of monocytes and is associated with the functional and cognitive decline.

Key Words: Inflammation—Aging—Monocyte function—CMV—Cognitive—Mini-mental.

Aging is associated with the chronic increase in systemic inflammatory status (inflammaging) (1). Inflammatory disorders are a major cause of age-related diseases such as Alzheimer's and cardiovascular diseases, which are important causes of increased mortality in elderly adults (2,3). These illnesses have been associated with the chronic increase in soluble inflammatory and coagulation markers, T-cell phenotype alterations, and cytomegalovirus (CMV) infection (4–6). Recent studies have focused on developing an improved understanding of the changes in innate immune cells revealing profound deregulations in the blood mononuclear phagocyte system with aging (7). Human monocytes are innate immune cells that are characterized by a high degree of heterogeneity and complexity. Monocytes include three subsets: the classical ($CD14^{++}CD16^{-}$), the intermediate ($CD14^{++}CD16^{+}$), and the nonclassical ($CD14^{dim}CD16^{+}$); they differ in size, morphology, phenotype, and function. The classical and intermediate subsets secrete high levels of proinflammatory cytokines in response to microbial products, and the nonclassical subset appears to "patrol" the vessel walls (8). However, data on the phenotype and functional age-related changes in monocytes and the association of these parameters with soluble inflammatory and coagulation biomarkers are scarce.

CMV is extremely prevalent in older people, and this virus has been identified in areas of the brain in individuals affected by Alzheimer's disease as a likely cause of this type of dementia (9). Even when CMV is in a latent state, inflammatory processes are ongoing, and viral reactivation leads to cytokine accumulation, resulting in direct damage to neurons (10). In this regard, latent CMV has been shown to be highly immunostimulatory in monocytes and macrophages (11,12), which results in heightened levels of circulating inflammatory cytokines. Recently, it has been described that monocyte activation and recruitment is associated with Alzheimer's amyloid pathology in mouse models (13). Nonetheless, basic functionality studies of human monocytes in elderly adults and the functional relationship to CMV infection, which eventually leads to functional and cognitive impairment in elderly adults, are unknown.

Thus, the aim of the present study was to analyze the phenotype and functional changes in the three monocyte subsets with aging and to study the relationship of these changes with the high persistent soluble inflammatory state, latent CMV infection, and functional and neurocognitive decline in elderly adults.

Method

Study Participants

Consecutive nursing home residents from Seville (Spain), between May 26, 2014 and July 3, 2014, were asked to participate in the HELIOPOLIS cohort. The inclusion criteria were being aged 65 years or older and having a self-sufficient health status. The exclusion criteria included any of the following situations during the preceding 6 months: (i) clinical data indicating active infections, (ii) hospital admission, (iii) anti-tumor therapy, or (iv) any treatment that could influence the immune status (mainly corticosteroids). The elderly group (Elderly, $n = 20$) was compared with the young healthy volunteers, who made up the control group (Young, $n = 20$). Laboratory evaluations were performed at the Laboratory of Immunovirology, Institute of Biomedicine, Virgen del Rocío University Hospital in Seville (Spain). All necessary institutional or ethical review board approvals were obtained, and written informed consent was obtained from all study participants.

Lymphocyte Count

The absolute $CD4^{+}$, $CD8^{+}$ T-cell counts (cells/mm^3) and the CD4:CD8 ratio were determined using an Epics XL-MCL flow cytometer (Beckman-Coulter, Brea, CA) according to the manufacturer's instructions.

Immunophenotyping and Intracellular Cytokine Staining of Monocytes

One milliliter samples of peripheral fresh whole blood samples were collected in ethylene diamine tetra-acetic acid tubes (within 30 min prior to the assay). Erythrocytes were lysed according to the manufacturer's instructions (Lyse Buffer, R&D, San Diego, CA), and the cells were immediately immunophenotyped using a panel of antibodies for lineage, activation, cell adhesion surface markers, and viability dye to exclude nonviable cells: LIVE/DEAD fixable Violet Dead Cell Stain, CD8-Qdot605, CD14-Qdot655, CD19-PB, and CD40-APC (Life Technologies, Carlsbad, CA); CD3-APC-H7, CD4-BV786, CD56-PB, CD16-PE-CF595, CD11b-Alexa700, CD62L-PE, and CD49d-FITC (BD Biosciences, Franklin Lakes, NJ); and HLA-DR-BV570, CD38-PerCPCy5.5 and CD163-PCy7 (Biolegend, San Diego, CA). Isotype controls for CD14 (Life Technologies), CD16, CD11b, CD62L, and CD49d (BD Biosciences) and CD38 and CD163 (Biolegend) were included in each experiment.

Intracellular cytokine staining was performed on 1.5 ml of whole blood that was collected in ethylene diamine tetra-acetic acid tubes. Erythrocytes were lysed according to the manufacturer's instructions (Lyse Buffer); the cells were washed twice with the washing buffer provided by the kit and were then washed with phosphate-buffered saline without calcium and magnesium and a maximum endotoxin level of 0.005 EU/ml. Then, the cells were resuspended in R10 media (RPMI 1640 supplemented with 10% heat-inactivated calf serum, 100 U/ml penicillin G, 100 µl/ml streptomycin sulfate, and 1.7 mM sodium glutamine) that contained 10 U/ml DNase I (Roche Diagnostics, Mannheim, Germany) and

rested for 1 hour before use. The cells were stimulated with 1 ng/ml lipopolysaccharide (LPS; Toll-like receptor [TLR] 4 agonist) or 20 ng/ml lipomannan from *Mycobacterium smegmatis* (LM-MS; TLR2 agonist), (both from InvivoGen, San Diego, CA) for in vitro stimulation or without stimuli for ex vivo results in the presence of 1 μg/ml of anti-CD28, 1 μg/ml of anti-CD49d (BD Biosciences), and 10 μg/ml of brefeldin A (Biolegend) at 37°C/5% CO_2 for 6 hours. Surface staining was performed for 20 minutes at room temperature using the following: LIVE/DEAD fixable Violet Dead Cell Stain, CD8-Qdot605, CD14-Qdot655, and CD19-PB (Life Technologies); CD56-PB and CD16-PE-CF595 (BD Biosciences); and HLA-DR-BV570 (Biolegend). The cells were washed and permeabilized using a Cytofix/Cytoperm kit (BD Biosciences) and stained with the following conjugated antibodies for 20 minutes at room temperature: CD3-APC-H7, interleukin-6 (IL-6)-PE, IL1-α-FITC, and tumor necrosis factor-alpha (TNF-α)-Alexa700 (BD Biosciences); and IL-1β-Alexa647, IL-8-PerCP, and IL-10-PCy7 (Biolegend). The cells were fixed with 4% paraformaldehyde. Isotype controls for CD14 (Life Technologies); CD16, TNF-α, IL-6, and IL1-α (BD Biosciences); and IL-1β, IL-8, and IL-10 (Biolegend) were included in each experiment. Monocytes were defined as high Forward/Side scatter and expressed HLA-DR, CD14, and/or CD16 but not CD3, CD8, CD19, or CD56. The cells were analyzed using a LSRFortessa Cell Analyzer (BD Biosciences). A minimum of 2,000,000 total events and 20,000 monocytes were recorded in each tube.

Assay of Soluble Biomarkers and Anti-CMV Titers

Sera samples were collected in serum separation tubes, and plasma samples were collected in ethylene diamine tetra-acetic acid tubes. The levels of high-sensitivity CRP (hsCRP) and β2-microglobulin (β2M) were determined with an immunoturbidimetric sera assay using Cobas 701 (Roche Diagnostics, GmbH [Mannheim, Germany]). The D-dimer levels were measured with an automated latex enhanced immunoassay using plasma samples (HemosIL D-Dimer HS 500, Instrumentation Laboratory). The sera and plasma samples were aliquoted and stored at −20°C until subsequent analysis of the following biomarkers: IL-6, IL-8, IL-10, IL-1β, and TNF-α (R&D Systems, Minneapolis, MN), sCD14, (Diaclone, Besançon, France) as well as sCD163 (Macro CD163 IQproducts, The Netherlands). Additionally, anti-CMV immunoglobulin G (IgG) titers were assayed using a CMV IgG enzyme-linked immunosorbent assay (GenWay, San Diego, CA).

Clinical Tests

Cognitive decline and functional decline were assessed using the Mini-Mental State Examination (MMSE) (14) and the Barthel Index of Activities of Daily Living (15) score tests, respectively. These tests were determined by geriatricians (R.R. and M.I.G., respectively) who considered sensory and motor functioning. The researchers who conducted the laboratory determinations were blinded to the clinical data until the statistical analysis.

Statistical Analysis

Continuous variables are expressed as medians and interquartile ranges, and categorical variables are expressed as percentages. The correlation between the variables was assessed using the Spearman rank test. The Mann–Whitney U test was used to analyze differences between unpaired groups. Differences between paired samples were determined by the Wilcoxon signed rank test. All p values less than .05 were considered significant. Variables with a p value less than .1 in the univariate analysis were entered into a stepwise multivariate logistic regression model to determine the associations with anti-CMV IgG levels and the mini-mental test results after controlling for age, gender, CD4 and CD8 T-cell count, the CD4:CD8 ratio, the simple and multiple intracellular expression of cytokines ex vivo, and soluble inflammatory markers. Statistical analysis was performed using the Statistical Package for the Social Sciences software (SPSS 17.0; SPSS, Chicago, IL). Prism, version 5.0 (GraphPad Software, Inc.) was used for the generation of the graphs. We defined polyfunctionality as the percentage of monocytes that produce multiple cytokines. Polyfunctionality pie charts were constructed using Pestle, version 1.6.2 and Spice, version 5.2 (both provided by M. Roederer, NIH, Bethesda, MD) (16).

Results

Demographic, Hematologic, and Serologic Characteristics of the Study Populations

There were 20 elderly participants (9 men and 11 women, aged 83 [67–90] years) who lived at the Heliopolis nursing home (Seville, Spain) and 20 young volunteers as the control group (14 men and 6 women, aged 35 [27–40] years) enrolled in the study. The following results were obtained from the elderly and young control groups, respectively: the median CD4 T-cell count was 666 [416–984] vs 753 [583–1040] (cells/mm^3); the CD8 T-cell count was 459 [305–812] vs 467 [308–619] (cells/mm^3); the CD4:CD8 ratio was 1.4 [1–2.1] vs 1.7 [1.2–2.9]; and the levels of anti-CMV were 19.0 [9.9–27.2] vs 7.3 [0.0–18.2] (IU/mm^3). No statistically differences were obtained in any of these variables, with the exception of the anti-CMV IgG levels, which were higher in the elderly group than in the young group (p = .009), as shown in Supplementary Figure 1. Of the 40 participants, 100% were considered CMV-seropositive (exhibiting signs of prior exposure) using the clinical cutoff point designated by the enzyme-linked immunosorbent assay test kits.

Monocyte Counts and the Frequency of the Activation of Phenotype Markers on Monocyte Subsets

The absolute total monocyte count was 455 [393–623] vs 330 [295–395] (cells/mm^3), and the absolute classical monocyte count was 204 [167–276] vs 285 [205–377] (cells/mm^3) in the elderly and young control groups, respectively. The total and classical absolute monocyte count was higher in the elderly group compared with that in the young group (p < .001, p = .024, respectively). We did not find differences in the frequency of monocyte subsets between groups. The percentage of activation and cell adhesion markers CD11b, CD40, CD49d, CD62L, CD163, and CD38 were similar in both groups regarding the three subsets. (Supplementary Table 1)

Intracellular Cytokine Production of Monocyte Subsets Ex Vivo and In Vitro Was Higher in the Elderly Group

Single cytokine production of monocytes

We addressed the characterization of the functionality, represented as IL1-α, IL1-β, IL-6, IL-8, IL-10, and TNF-α intracellular production ex vivo and responsiveness in vitro to LM-MS (TLR2 agonist)

and LPS (TLR4 agonists) of the three monocyte subsets. Note that after LPS stimulation, despite the shedding of CD16, the nonclassical subset can be perfectly gated (Figure 1A and B).

Ex vivo, the elderly group showed a significantly higher percentage of intermediate monocytes that produced all of the cytokines described above. The results were similar for the other two subsets with the exception of IL10, whose levels did not differ between the subsets and TNF-α and whose levels were similar for classical monocytes in each group (Table 1 and Supplementary Figure 2). In vitro, the elderly group showed a significantly higher percentage of classical monocytes that expressed intracellular IL1-β and a higher percentage of the intermediate subset that expressed IL1-α, IL1-β, IL-8, and TNF-α in response to LM-MS. No significant differences were observed in the nonclassical subset in response to LM-MS (Table 1). Regarding LPS stimulation, although we did not observe significant differences between the groups, which was most likely due to the high dispersion of the values, it is important to note that the elderly group exhibited higher responsiveness in terms of almost all of the cytokines that showed twofold to threefold median increases compared with the young group in the three monocyte subsets.

Multiple Cytokine Production of Monocytes

We also analyzed the characterization of simultaneous multiple cytokine expression per cell, which is also termed polyfunctionality. The classical subset showed higher polyfunctionality distribution ex vivo in the elderly participants (data not shown). After stimulation with LPS, the classical monocytes in the elderly group expressed significantly higher levels of multiple cytokines compared with the young group (Figure 1C). No differences were observed for the other monocyte subsets either after LM-MS stimulation or based on the ex vivo results. Nonetheless, a more in-depth analysis of the 63 possible multiple cytokine combinations ex vivo revealed that the specific proinflammatory cytokine combination of cells expressing IL1-α^+, IL1-β^+, IL-6^+, IL-8^+, IL-10^-, and TNF-α^+ (a+b+6+8+10-T+) was higher in the classical and intermediate monocytes of the elderly group (p = .014 and p = .015, respectively). Additionally, the elderly group showed a higher percentage of nonclassical monocytes that simultaneously produced IL1-α^+, IL1-β^+, IL-6^-, IL-8^+, IL-10^+, and TNF-α^+ (a+b+6–8+10+T+; p = .048). Significantly more combination of four, three, four, and five cytokines occurred in the three monocyte subsets in the elderly group (data not shown). The detailed analysis of the in vitro responsiveness revealed similar results; the classical and intermediate monocytes exhibited a greater polyfunctional proinflammatory response to LM-MS and LPS (data not shown).

Single and Multiple Intracellular Functionality of Monocytes Is Associated With Higher Soluble Levels of Inflammatory and Coagulation Biomarkers

We determined the soluble concentrations of inflammatory and coagulation biomarkers. The IL-1β and IL-10 levels were below the detection limit of the assay. Consistent with previous data (1), the elderly group exhibited higher soluble levels of IL-8, IL-6, TNF-α, hsCRP, β2M, and D-dimer compared with the young control group (Supplementary Table 2). Interestingly, we found that the ex vivo

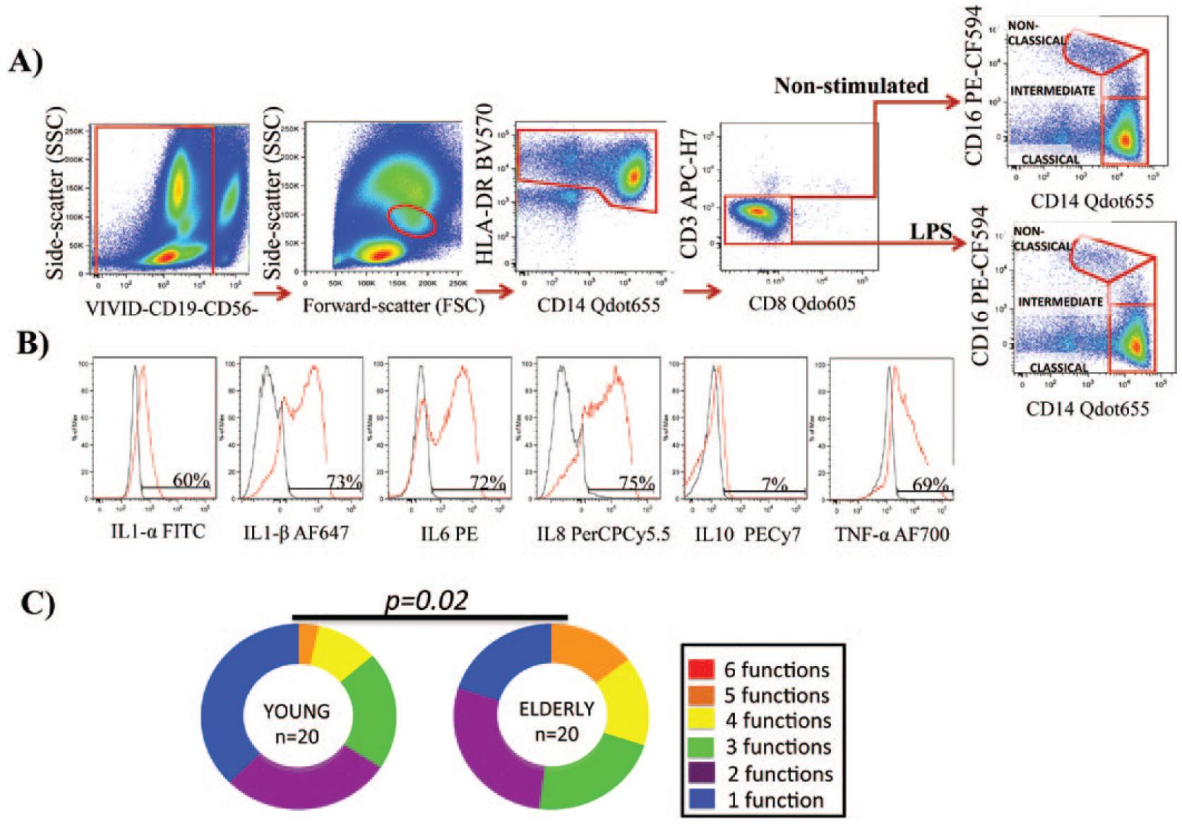

Figure 1. (A) Schematic diagram of the gating strategy of an elderly participant. Note that after LPS stimulation, despite the shedding of CD16, the nonclassical subset was gated. (B) Histogram representation of cytokine expression on classical monocytes without stimuli and with LPS stimulation. (C) Pie charts show classical monocytes with up to six functional responses to LPS stimulation. Statistically significant differences between the findings are shown. Pestle version 1.6.2 and Spice version 5.2 (M. Roederer, NIH, Bethesda, MD) were used.

Table 1. Percentage of Each Monocyte Subset that Produces Different Intracellular Cytokines Ex Vivo and In Vitro

	Elderly Group (n = 20)			Young Group (n = 20)			p Values	p Values	
	Ex Vivo	In Vitro		Ex Vivo	In Vitro		Ex Vivo	In Vitro	
		LMMS	LPS		LMMS	LPS		LMMS	LPS
Classical monocytes									
IL1-α	0.36 (0.07–1.65)	0.70 (0.09–3.42)	7.35 (0.37–21.26)	0.04 (0.02–0.11)	0.09 (0.02–0.50)	1.42 (0.62–6.59)	.001**	.060***	.200
IL1-β	0.39 (0.19–1.45)	8.00 (3.13–15.49)	35.45 (18.89–54.20)	0.13 (0.06–0.29)	3.12 (1.26–5.82)	13.45 (4.33–34.88)	.001**	.003**	.068***
IL-6	0.59 (0.31–1.17)	4.16 (2.50–8.97)	23.35 (10.54–39.28)	0.25 (0.10–0.47)	3.05 (1.58–6.73)	14.15 (4.47–34.53)	.002**	.140	.240
IL-8	2.26 (1.41–4.13)	15.30 (8.19–27.94)	44.19 (19.07–62.32)	1.15 (0.49–1.62)	12.63 (4.55–18.90)	26.18 (11.85–58.81)	.007**	.080***	.200
IL-10	0.11 (0.04–0.50)	0.03 (0.00–0.20)	0.01 (0.00–1.33)	0.18 (0.04–0.75)	0.02 (0.00–076)	0.05 (0.00–0.81)	.758	.779	.841
TNF-α	0.27 (0.14–0.42)	1.44 (0.58–2.65)	12.64 (4.14–25.52)	0.20 (0.08–0.30)	0.93 (0.39–1.94)	6.80 (1.69–15.00)	.289	.140	.140
Intermediate monocytes									
IL1-α	1.91 (0.55–4.39)	2.54 (0.37–5.49)	15.54 (3.30–26.96)	0.18 (0.04–0.40)	0.19 (0.01–0.61)	1.86 (0.50–7.87)	<.001*	.001**	.086***
IL1-β	1.37 (0.57–3.85)	9.29 (4.00–15.08)	32.71 (16.84–46.76)	0.56 (0.18–0.78)	2.86 (1.48–8.70)	10.56 (3.25–29.67)	.004**	.007**	.072***
IL-6	1.28 (0.67–2.67)	5.86 (3.69–11.42)	24.55 (8.89–40.95)	0.62 (0.22–1.61)	4.45 (1.88–7.80)	11.00 (4.76–21.62)	.021**	.211	.192
IL-8	4.42 (1.99–7.58)	14.24 (10.94–24.72)	36.35 (15.88–51.62)	0.77 (0.43–3.86)	10.83 (2.63–18.52)	21.44 (7.18–37.25)	.003**	.049**	.157
IL-10	2.10 (0.58–4.39)	0.10 (0.00–1.69)	0.14 (0.00–1.59)	0.41 (0.06–1.56)	0.05 (0.00–0.78)	0.11 (0.00–1.64)	.004**	.738	.904
TNF-α	0.57 (0.39–1.07)	3.14 (1.33–5.54)	13.02 (2.75–29.16)	0.30 (0.12–0.61)	0.89 (0.53–2.65)	4.49 (1.94–14.00)	.026**	.030**	.183
Nonclassical monocytes									
IL1-α	1.10 (0.56–2.12)	0.33 (0.00–2.00)	0.48 (0.00–0.88)	0.31 (0.15–0.55)	0.35 (0.00–1.06)	0.30 (0.00–1.55)	<.001*	.841	.947
IL1-β	1.52 (0.75–3.90)	1.73 (0.51–4.97)	1.69 (0.00–5.00)	0.50 (0.18–1.22)	1.05 (0.50–3.43)	1.10 (0.16–1.93)	.001**	.565	.738
IL-6	0.87 (0.23–1.83)	0.81 (0.08–2.58)	0.92 (0.11–3.03)	0.23 (0.08–0.83)	0.31 (0.00–1.24)	0.16 (0.02–1.62)	.068***	.341	.192
IL-8	6.81 (2.35–11.78)	1.19 (0.00–4.21)	1.70 (0.16–6.35)	1.45 (0.70–6.67)	1.23 (0.37–4.29)	3.45 (0.32–6.38)	.038**	.583	.565
IL-10	4.21 (1.05–9.97)	0.77 (0.00–2.08)	0.00 (0.00–1.63)	2.37 (1.04–5.16)	0.40 (0.14–2.24)	0.60 (0.00–1.96)	.327	.678	.211
TNF-α	2.43 (1.14–3.91)	1.67 (0.23–3.31)	1.56 (0.18–3.60)	1.12 (0.54–1.63)	1.17 (0.32–4.78)	1.03 (0.22–4.04)	.010**	.883	.925

Notes: Values are expressed as the median and interquartile range. Mann–Whitney U test was used to compare the values of the elderly group with those of the the young control group.
*p < .001. **p < .05. ***p < .1.

single cytokine production was positively and strongly correlated with soluble inflammatory levels in the three monocyte subsets. Remarkably, the β2M and D-dimer levels were strongly and positively associated with the expression of almost all of the intracellular cytokines in the three monocyte subsets (Figure 2). When this analysis was applied to multiple cytokine combinations, in the classical subset, the ex vivo polyfunctionality was positively correlated with the soluble inflammatory markers, especially with β2M and D-dimer (Supplementary Figure 3). Similar results were obtained for the intermediate and nonclassical subset (data not shown).

Regarding the phenotype activation markers, we also found strong associations between activation cell surface markers on monocytes and soluble inflammatory concentrations (data not shown). Of special interest were the associations of the scavenger receptor CD163 with the following in the elderly group: sCD14 (p = .045; ρ = .465), IL-6 (p = .020; ρ = .542), hsCRP (p = .029; ρ = .500), and β2M (p = .004; ρ = .634).

Monocyte Activation Phenotype Is Associated With Anti-CMV Titers and Functional and Cognitive Decline in the Elderly Group

The relationship between monocyte activation and anti-CMV IgG antibodies in the elderly group was analyzed. Soluble CD163 was positively associated with anti-CMV IgG levels (p = .007, ρ = .579) in the elderly group. Regarding surface expression, CD62L in the classical subset was inversely and independently associated with anti-CMV IgG levels (Table 2) after adjusting for age, gender, CD4 and CD8 T-cell count, the CD4:CD8 ratio, the ex vivo single and multiple intracellular production of cytokines, and soluble inflammatory markers. Figure 3A and B shows the graphical representation of the correlations between anti-CMV IgG levels and CD40 (p = .005, ρ = .618) and CD62L (p = .013, ρ = –.561) surface expression on classical monocytes. On the contrary, there was no association between those parameters in the young group.

We were interested in analyzing which factors are associated with the functional and cognitive decline. According to the Mahoney and Barthel definition of the test, we categorized the highest Barthel score (100), which means that the person requires no assistance with any aspect of the tasks of the test as functional independence (n = 11/20). If the Barthel score was below 100, then we categorized the person as no functional independence (n = 9/20). According to these groups, we found that the CD163 and CD38 surface expression on classical monocytes was higher in the functional independence group (p = .041, p = .026, respectively). There were no differences in gender, CD4 and CD8 T-cell count, CD4:CD8 ratio or anti-CMV IgG levels between the categories. We also examined the relationship of the immunological variables and monocyte markers with the cognitive decline assessed with the MMSE test. Only the expression of CD11b in the classical subset and the CD4:CD8 ratio were independently associated with the MMSE (Table 3) results after adjusting for age, gender, CD4 and CD8 T-cell count, the CD4:CD8 ratio, the ex vivo single and multiple intracellular production of cytokines, and the soluble inflammatory markers. Figure 3C shows the graphical representation of the correlations between the MMSE and the CD11b expression on classical monocytes in the elderly group.

Discussion

The results presented herein comprehensively characterize a higher single and multiple intracellular cytokine production by monocytes both ex vivo and in vitro in the elderly group. Moreover, the ex vivo cytokine expression together with phenotype activation of surface markers of monocytes was strongly associated with soluble inflammatory biomarkers, such as IL-6, D-dimer, hsCRP, and sCD14, which are all known to be associated with increased morbidity risk. In addition to these findings, we showed that phenotype activation markers of monocytes are associated with anti-CMV IgG levels and with the functional and cognitive decline in the elderly group.

The present study adds novel data to the hyperactivation and functional deregulation of monocytes with aging. We carefully characterized the intracellular cytokine responsiveness ex vivo and to TLR-2 and TLR-4 agonists in vitro and showed that

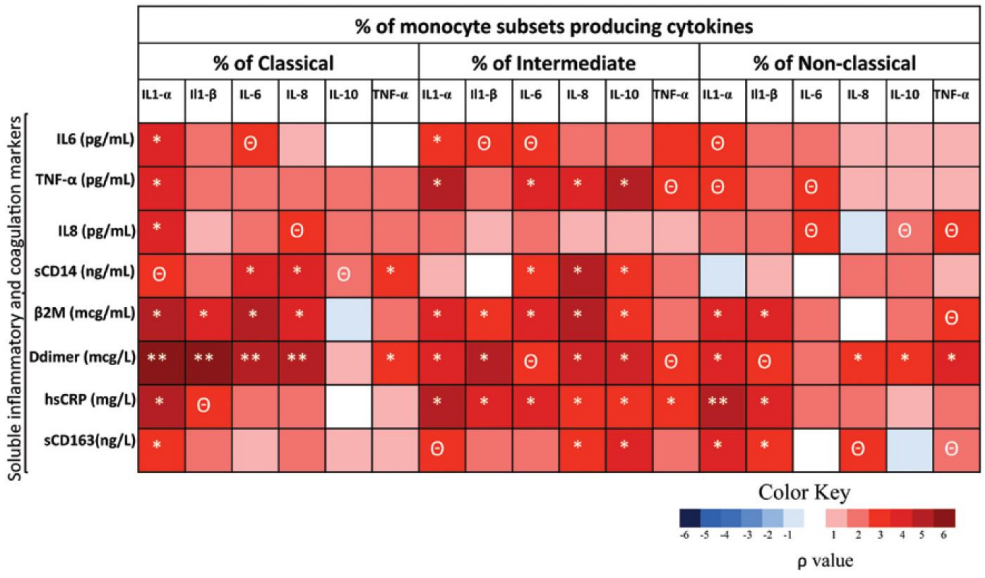

Figure 2. Correlations among the soluble biomarkers and monocyte function ex vivo in the elderly and young groups (n = 40). Heatmap representing negative (blue shading) and positive (red shading) associations between soluble inflammatory markers and single intracellular cytokine production in the three monocyte subsets. Spearman ρ correlation coefficient test was used. $^{**}p < .001$. $^{*}p < .05$. $^{\ominus}p < .1$.

Table 2. Relationship Between Immunological Variables and Monocyte Markers With Anti-CMV IgG Levels in the Elderly Group

	Univariate			Multivariate		
	β	CI (95%)	*p* Value	β	CI (95%)	*p* Value
% CD11b expression on classical	1.779	−0.127 to 3.685	.065			NS
% CD40 expression on classical	0.385	0.169 to 0.601	.002			NS
% CD62L expression on classical	−1.179	−1.97 to −0.387	.006	−1.477	−2.256 to −0.698	.001
CD4:CD8	−7.56	−16.193 to −1.073	.082			NS

Note: CI = confidence interval; NS = Nonsignificant. Stepwise regression model.

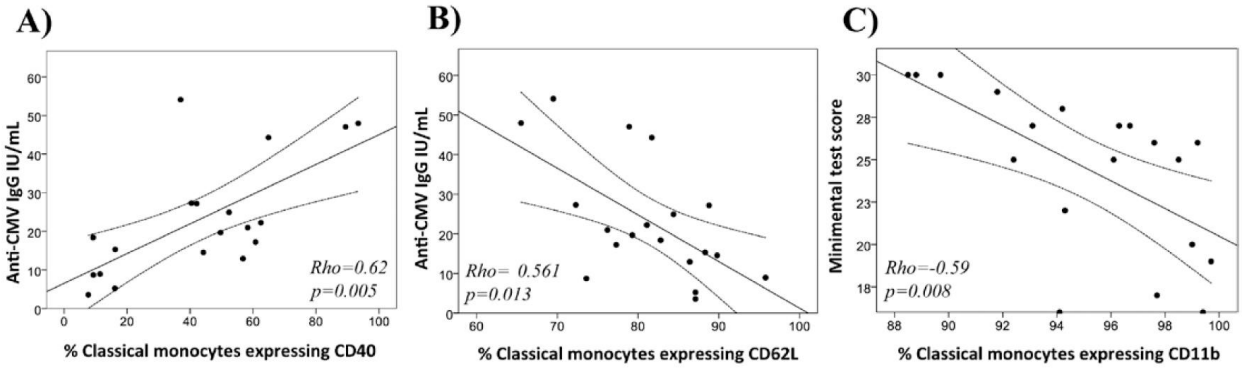

Figure 3. (A and B) Graphical representation of the relationship between anti-cytomegalovirus titers and the percentage of monocytes expressing CD40 and CD62L. (C) Graphical representation of the association between Mini-Mental State Examination and the CD11b expression on classical monocytes in the elderly group. Spearman ρ correlation coefficient test was used.

the responsiveness was higher in the elderly group. The nonclassical subset showed the lowest responsiveness to TLR-2 and TLR-4 stimulation according to previous data (8). In addition, ex vivo, this subset expressed the highest levels of TNF-α and IL-10, and these data support the evidence that the three monocyte subsets are not only phenotypically different but also functionally different.

We also characterized the polyfunctionality changes of these cells with advanced age. We interestingly found that multiple cytokine distribution was higher in the classical subset ex vivo and in response to LPS with aging. Most importantly, the expression of both single and combinations of multiple intracellular cytokines ex vivo was strongly associated with soluble inflammation and coagulation biomarkers, which suggests that monocytes are the key cell source of the chronic inflammatory state defined in aging. In the T-cell scenario, immunosenescence is related to the changing phenotypic composition of T cells with age. Impaired T-cell polyfunctionality in older adults have been associated with an increased 2-year mortality rate (17) and leads to diminished immune responses following vaccination (18). In the HIV field, enhanced polyfunctionality in HIV-1–specific $CD8^+$ T cells has been associated with superior control of the virus (19). Our results demonstrate that monocyte polyfunctionality in the elderly group is not desirable because it is correlated with soluble inflammatory markers that are known to be associated with increased morbidity and mortality risk.

Mortality and morbidity in the elderly group are associated with impaired innate immunity to pathogens, CMV infection, and a persistent low-grade soluble inflammatory state (2,5,20). The present findings are the first to demonstrate the relationship between monocyte activation, anti-CMV IgG titers, and functional and cognitive decline in the aged population. CD62L is an adhesion molecule that is responsible for monocyte rolling and adhesion to endothelial cells. We found that the expression of CD62L on classical monocytes was strongly and inversely associated with anti-CMV IgG levels, which is even more consistent and profound than the CD4:CD8 ratio, a well-established marker of immunosenescence. The reduced surface expression of CD62L on monocytes with aging has been described by other authors (21). The downregulation of CD62L is believed to impair rolling and may increase firm attachment of the cells to vessels, favoring endothelial migration. This mechanism may therefore promote atherosclerotic plaque formation and other inflammatory diseases in the elderly population.

Cognitive and functional impairments are important causes of increased morbidity and mortality in older people. Researchers agree that understanding the mechanisms to establish early diagnosis and treatment benefits is critical. We found that the phenotype activation of monocytes is strongly associated with functional and cognitive decline in the elderly group. Our results provide a more complete understanding of the mechanisms that contribute to the neuropathogenesis of age-related diseases. Moreover, we showed that the expression of the CD11b integrin, which pairs with CD18 to form a heterodimeric type 1 transmembrane receptor (CD11b/CD18; β2αM) on classical monocytes (an easily accessible biomarker), was strongly associated with the rate of cognitive decline in the elderly group, in addition to the currently used T-cell markers. Recently, the upregulation of other cell adhesion surface markers, such as the chemokine receptor 2 (CCR2) on $CD16^+$ monocytes, has been associated with HIV-associated neurocognitive disorders in the premature immunosenescence of HIV-infected patients (22). Our results provide more evidence that the activation of monocytes may significantly contribute to the development of age-related diseases.

The potential mechanisms that drive these phenotypes of the hyperactivation and high inflammatory ex vivo and in vitro state of monocytes could involve persistent viral infections such as CMV that stimulate and exhaust the immune system. CMV is thought to persist

Table 3. Relationship Between Immunological Variables and Monocyte Markers With Mini-mental Test Score in the Elderly Group

	Univariate			Multivariate		
	β	CI (95%)	*p* Value	β	CI (95%)	*p* Value
% CD11b expression on classical	−0.815	−1.34 to −0.29	.004	−0.888	−1.42 to −0.35	.003
Age	−0.213	−0.38 to −0.046	.015			NS
% CD40 expression on classical	−0.083	−0.17 to −0.002	.046			NS
% CD62L expression on classical	0.291	0.014 to 0.567	.041			NS
Anti-CMV IgG	−0.132	−0.27 to 0.012	.070			NS
CD4:CD8	3.807	1.493–6.121	.003	2.769	0.873–4.666	.007

Note: CI = confidence interval. Stepwise regression model.

in a latent state with its DNA genome harbored primarily in monocytes (23), which establishes a chronic infection with intermittent reactivations. CMV reactivations and new reinfections that occur more frequently in the elderly group (24) than in the young might be a driving force in the inflammatory state and the activation of monocytes/macrophages (11,12). In vitro studies demonstrated that upon infection with other viruses, these cells mediated the differentiation of resting B cells to plasmablasts as well as IgG antibodies via BAFF/APRIL and IL-10 signaling (25). Hence, this could be the linking mechanism between monocyte activation and the demonstrated enhanced anti-CMV IgG levels. However, we cannot exclude other possible causes such as microbial translocation caused by barrier defects in gut-associated lymphoid tissue related to advanced age, reactivation of endogenous pathogens such as *Mycobacterium tuberculosis* and other herpesviruses.

Our study was limited by the small numbers of participants, and we did not have sufficient power to statistically assess the relationship between the enhanced CD11b expression on monocytes for the diagnoses of cognitive impairment and the decreased CD38 and CD163 expression on monocytes for the diagnoses of functional impairment. CD163 is a member of a scavenger receptor super family and is a specific monocyte and macrophage activation marker. The shedding upon activation of these cells leads to the reduced CD163 surface expression, and the higher levels of the soluble form of CD163 have been associated with age-associated diseases (26,27). Future studies with larger cohorts will be performed to more clearly evaluate the associations among phenotype changes and worse clinical outcomes. Other limitation of our data is the fact that all young controls were CMV seropositive, this makes impossible to analyze separately the effect of age and CMV infection in our study. In our previous work (28), we described 82% prevalence of CMV seropositivity in young controls with median age of 33 years, 2 years younger than the median age in this work, these discrepancies may be due to different sensitivity of the kits used for determinations. Additionally, more in vitro and in vivo longitudinal studies should be performed to analyze the underlying mechanisms in more detail and to develop approaches for determining the cause and effect relationship among CMV infection, the age-related monocyte changes, and clinical impairment.

In summary, our results demonstrate that monocytes are key cell candidates for the potential source of the high soluble inflammatory levels of aged participants. Our findings suggest that CMV infection might be a driving force in the activation of monocytes shown to be associated with the functional and cognitive decline in the elderly group.

Supplementary Material

Supplementary material can be found at: http://biomedgerontology.oxfordjournals.org/

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Phenotype and polyfunctional deregulation involving IL-6 and IL-10-producing monocytes of HIV-infected patients on cART differ from those of aging

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Title: Phenotype and polyfunctional deregulation involving IL-6- and IL-10-producing monocytes of HIV-infected patients on cART differ from those of aging

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ABSTRACT:

Background: Despite the relevance of monocytes as promoters of the inflammatory response, whether HIV infection induces premature age-related changes to the phenotype and function of monocytes or these alterations are different and/or specifically driven by HIV remains to be mechanistically determined.

Methods: We assayed the activation phenotype and the responsiveness in vitro to TLR agonists in classical, intermediate and non-classical subsets of monocytes by assessing the intracellular IL-1α, IL-1β, IL-6, IL-8, TNF-α and IL-10 production in patients on cART (n=20, HIV) compared to two groups of uninfected controls (age-matched, n=20, Young or over 65 years old, n=20, Elderly).

Results: HIV-infected patients showed a more activated phenotype of monocytes than the elderly. Regarding functionality, under unstimulated conditions, the HIV group showed a higher percentage of classical monocytes producing IL-6 and IL-10 compared with controls. The percentage of cells with multiple cytokine production (polyfunctionality) in response to TLR agonists in the HIV group, specially with IL-10, was higher than in the controls.

Conclusion: Inflammatory alterations associated with monocytes in HIV infection are different from those of aging. This monocyte dysfunction, mainly characterized by high levels of IL-6- and IL-10-producing monocytes, may have clinical implications in HIV-infected patients different from those of aging.

Key words: HIV, inflammation, monocyte, aging

INTRODUCTION

Aging and HIV infection are associated with profound changes in the immune system, inducing several similar defects associated with T-cell function [1, 2]. Additionally, higher levels of inflammatory and coagulation biomarkers have been reported for HIV-infected patients on combined antiretroviral therapy (cART) and for elderly uninfected subjects [3-5], which likely increase their risks for developing chronic diseases [6, 7]. However, we have recently shown marked differences in the inflammatory biomarker profile of cART-treated and HIV-uninfected elderly subjects [8].Together, these findings beg the question of how the inflammatory mechanisms of HIV infection and age intersect. In this sense, the latest studies have focused on better understanding the changes in innate immune cells, revealing profound deregulations in the blood mononuclear phagocyte system with aging and HIV infection [9, 10].

Human monocytes are innate immune cells characterized by a high degree of heterogeneity and complexity. Monocytes comprise three subsets, the classical monocytes (CD14++CD16−), the intermediate monocytes (CD14++CD16+), and the non-classical monocytes (CD14dimCD16++), that differ in size, morphology, phenotype and function. The classical and intermediate subsets secrete high levels of pro-inflammatory cytokines in response to microbial products, and the non-classical subset appears to patrol the vessel wall [11]. However, despite the fact that monocytes are potentially the major source of the circulating inflammatory cytokines found in HIV-infected patients and normal aging [12], mechanistically, the information about the single and multiple cytokine production (polyfunctionality) of these cells in both scenarios has not been explored.

The aim of this study was to comprehensively analyze phenotypic and functional characteristics of the three subsets of monocytes in cART-treated HIV infected patients and to compare them to those from the aging scenario.

PATIENTS AND METHODS

Study participants

Twenty Caucasian, asymptomatic, HIV-infected patients on suppressive cART (defined as persistent undetectable viral load for at least 6 months) that consecutively visited the outpatient clinic of the Infectious Disease Unit at Virgen del Rocío University Hospital in Seville (Spain) between May and July of 2014 were included. Patients were excluded if they had a current or previous history of cardiovascular disease, diabetes mellitus, chronic kidney disease or had reached clinical category C of HIV infection.

HIV-infected patients (the HIV group, n=20) were compared with age-matched, HIV-uninfected healthy subjects, called the Young group (Young, n=20), and with elderly subjects, called the Elderly group (Elderly, n=20), who had already been studied with a different objective [12] and served as the control groups for the present study. Briefly, consecutive nursing home residents from Seville (Spain) were asked between May and July of 2014 to participate in this cohort. The inclusion criteria were being aged 65 years or older and having a self-sufficient health status. The exclusion criteria included any of the following situations during the preceding six months: (1) clinical data indicating active infections, (2) hospital admission, (3) anti-tumor therapy, or (4) any treatment that could influence the immune status (mainly corticosteroids). Laboratory evaluations were performed at the Laboratory of Immunovirology, Institute of Biomedicine (IBiS), Virgen del Rocío University Hospital in Seville (Spain). All necessary institutional or ethical review board approvals were obtained, and written informed consent was obtained from all study participants.

Laboratory methods

The absolute CD4+, CD8+ T-cell counts (cells/mm^3) and the CD4:CD8 ratio were determined using an Epics XL-MCL flow cytometer (Beckman-Coulter, Brea, CA, USA) according to the manufacturer's instructions. The plasma HIV-1 RNA concentration (HIV-RNA copies/mL) was measured using quantitative polymerase chain reaction (COBAS Ampliprep/COBAS Taqman HIV-1 Test, Roche Molecular Systems, Basel, Switzerland) according to the manufacturer's protocol. The detection limit for this assay was 20 HIV-RNA copies/mL. Plasma samples were tested for an HBV-related marker (HBsAg) using an HBV enzyme-linked immunosorbent assay

(ELISA) (Siemens Healthcare Diagnosis, Malvern, PA). HCV exposure (anti-HCV) was detected using an HCV enzyme-linked immunosorbent assay (ELISA) (Siemens Healthcare Diagnosis, Malvern, PA).

Immunophenotyping and intracellular cytokine staining of monocytes

The methods for immunophenotyping and intracellular cytokine staining of monocytes have previously been described in detail [12]. Briefly, samples consisting of one mL of peripheral fresh whole blood were collected in EDTA tubes (within 30 min prior to the assay). Erythrocytes were lysed according to the manufacturer's instructions (Lyse Buffer, R&D, CA), and the cells were immunophenotyped using a panel of antibodies for lineage, activation, cell adhesion surface markers and a viability dye to exclude nonviable cells: LIVE/DEAD, CD8, CD19, and CD40 (clone HB14) (Life Technologies, CA, USA); CD3, CD4, CD56, CD16 (clone 3G8), CD11b (clone ICRF44), CD62L (clone DREG-56) and CD49d (clone gF10) (BD Biosciences, NJ, USA); and CD14 (clone M5E2), HLA-DR (clone L243), CD38 (clone HB-7), and CD163 (clone GHI/G1), (Biolegend, CA, USA). Isotype controls for CD14 (Life Technologies, CA, USA); CD16, CD11b, CD62L and CD49d, (BD Biosciences, NJ, USA); and CD38 and CD163 (Biolegend, CA, USA) were included in each experiment. In vitro assays were performed on 1.5 ml of whole blood that was collected in EDTA tubes (within 30 min prior to the assay). Erythrocytes were lysed according to the manufacturer's instructions (Lyse Buffer, R&D, CA). Then, the cells were resuspended in R10 media (RPMI 1640 supplemented with 10% heat-inactivated calf serum, 100 U/ml penicillin G, 100 µl/ml streptomycin sulfate, and 1.7 mM sodium glutamine) that contained 10 U/ml DNase I (Roche Diagnostics) and rested for 1 h before use. Then, cells were stimulated with 1 ng/mL LPS ultrapure (Toll-like receptor (TLR) 4 agonist), 20 ng/mL lipomannan from *M. smegmatis* (LM-MS) (Toll-like receptor (TLR) 2 agonist) or 0.5 µg/mL ssRNA40s/Lyovec (TLR 7 agonist) (all from InvivoGen, San Diego, CA, USA) for six hours for in vitro stimulation or without stimuli for the unstimulated condition, in the presence of 1 µg/ml of anti-CD28, 1 µg/ml of anti-CD49d (BD Biosciences, NJ, USA), 10 µg/ml of brefeldin A (BFA) (Biolegend, CA, USA) at 37°C/5% CO_2 for six hours. Surface staining was performed using the following antibodies: LIVE/DEAD, CD8, CD14, and CD19 (Life Technologies, CA, USA); CD56 and CD16 (BD Biosciences, NJ, USA); and HLA-DR (Biolegend, CA, USA). The cells were washed and permeabilized using a Cytofix/Cytoperm kit (BD Biosciences) and

stained with the following antibodies: CD3, IL-6 (clone MQ2-6A3), IL-1α (clone AS5) and TNF-α (clone MAb11) (BD Biosciences, NJ, USA) and IL-1beta (β) (clone JK1B1), IL-8 (clone BHO814), and IL-10 (clone JE53-9D7), (Biolegend, CA, USA). Isotype controls for CD14 (Life Technologies, CA, USA); CD16, TNF-α, IL-6, and IL-1α (BD Biosciences, NJ, USA); and IL-1β, IL-8, and IL-10 (Biolegend, CA, USA) were included in each experiment. Monocytes were defined as high Forward (FSC)/Side scatter (SSC), and expressing HLA-DR, CD14 and/or CD16, but not CD3, CD8, CD19 or CD56. The cells were analyzed using a LSR Fortessa Cell Analyzer (BD Biosciences, NJ, USA). A minimum of 2,000,000 total events and 20,000 monocytes were recorded in each tube (Supplementary Figure 1). Data were analyzed using FlowJo 8.7.7 (TreeStar). We calculated the responsiveness of cells in vitro by subtracting the results of the unstimulated condition.

Assay of soluble biomarkers and anti-CMV titers.

Serum samples were collected in serum separation tubes, and plasma samples were collected in EDTA tubes. The levels of high-sensitivity CRP (hsCRP) and β2-microglobulin (β2M) were determined with an immunoturbidimetric serum assay using Cobas 701® (Roche Diagnostics, GmbH, Mannheim, Germany). The D-dimer levels were measured with an automated latex-enhanced immunoassay using plasma samples (HemosIL D-Dimer HS 500, Instrumentation Laboratory, USA). The serum and plasma samples were aliquoted and stored at -20°C until subsequent analysis of the following biomarkers: IL-6, IL-8, IL-10, IL-1β, and TNF-α (R&D Systems, Minneapolis, MN, USA), sCD14, (Diaclone, Besançon, France) and sCD163 (Macro CD163 IQ products, The Netherlands). Additionally, anti-CMV IgG titers were assayed in the sera using a cytomegalovirus IgG enzyme-linked immunosorbent assay (GenWay, San Diego, CA).

Statistical analysis

Continuous variables are expressed as medians and interquartile ranges (IQR), and categorical variables are expressed as percentages. The correlations between continuous variables were assessed using the Spearman rank test. The Mann-Whitney U test was used to analyze differences between unpaired groups. Statistical analyses were performed using the Statistical Package for the Social Sciences software (SPSS 17.0; SPSS, Chicago, IL). Prism, version 5.0 (GraphPad Software, Inc.) was used to generate the graphs. We defined polyfunctionality as the percentage of monocytes that produce

multiple cytokines. Polyfunctionality pie charts were constructed using Pestle, version 1.6.2 and Spice, version 5.2 (both kindly provided by M. Roederer, NIH, Bethesda, MD) [13].

RESULTS

Characteristics of the study subjects

The demographic and immunovirological characteristics of all study participants are shown in Table 1. The HIV and Young groups were predominantly men, while only 45% of the elderly subjects were male. The median age was 83 [67-90] years for this group. The absolute total monocyte counts in the HIV group were similar to the Elderly counts but higher than the Young counts. We did not find differences in the frequency of monocyte subsets between the groups (data not shown).Of the 60 participants, 100% were CMV-seropositive. Similar higher anti-CMV-IgG titers were found in the HIV and Elderly when compared to the Young group. The HIV group showed a median nadir CD4 T cell count of 330 [266-375] cells/mm^3, the median time since HIV+ diagnosis was 6 [3-9] years and the median time since HIV-RNA was undetectable in plasma was 6 [3-9] years. None of HIV-infected patients had histories of prior Category C clinical stage. Neither patients nor controls had prior HCV exposure (anti-HCV negative) and all of them were HBsAg negative.

HIV-infected patients showed a more activated phenotype of monocytes than the elderly

Figure 1 displays the percentage of surface expression of the activation and cell adhesion markers CD163, CD49d, CD62L, CD40, CD11b and CD38 in the three subsets of monocytes among the groups. The expression of the scavenger receptor CD163 in the HIV group was remarkably higher compared to both control groups in the three subsets of monocytes ($p<0.001$ for all comparisons). The expression of integrin CD49d, implicated in homing and stabilizing the adhesion of leukocytes to endothelial cells, was much higher in the HIV group compared to expression levels in both control groups in the classical and non-classical subsets ($p<0.001$ for all comparisons). Additionally, the HIV group expressed the highest levels of CD62L, implicated in homing and cell adhesion and migration, in the classical and intermediate monocytes ($p=0.03$ and $p=0.001$, respectively). The percentage of cells that expressed the costimulatory protein CD40 was significantly higher in the HIV group, compared to both control groups, only in the non-classical subset ($p<0.001$). The expression of CD11b, which mediates leukocyte adhesion and migration, was higher in the HIV group in the intermediate subset ($p=0.001$). No differences were found for CD38 expression among the groups.

HIV-infected patients showed higher levels of monocytes producing single and multiple cytokines under unstimulated conditions and in response to TLR agonists, when compared to elderly donors

We addressed the characterization of the functionality, represented as IL-1α, IL-1β, IL-6, IL-8, IL-10 and TNF-α intracellular production in the unstimulated condition, and the responsiveness to LM-MS (TLR2 agonist), LPS (TLR4 agonist) and ssRNA40 (TLR7 agonist) in vitro of the three monocyte subsets. Note, that the conditions utilized minimize the shedding of CD16 and allowed the identification of the non-classical subset (Supplementary Figure 1).

The HIV group showed a significantly higher percentage of the three monocyte subsets that produced IL-6 and IL-10 (Table 2) under unstimulated conditions. Similar results were observed for the median intensity fluorescence (MFI) of positive cells (Supplementary Table 1). Figure 2A shows the percentage of classical monocytes producing simultaneous single and multiple cytokines per cell under unstimulated conditions. For the multiple combination of six cytokines, IL-1α+, IL-1β+, IL-6+, IL-8+, IL-10+ and TNF-α+ are represented as, a+b+6+8+10+T+, single IL-6 production is represented as a-b-6+8-10-T- and IL-10 as a-b-6-8-10+T-. These functionalities were higher in the HIV group than in the controls (p<0.001, for all comparisons) (Figure 2A). The polyfunctional distribution of cytokines was different among groups (p<0.001, for all comparisons) in the unstimulated condition (Figure 2B). Remarkably prominent was the production of IL-10 in HIV-infected subjects (arcs in Figure 2B), particularly in the 1+ function. This high monofunctional distribution, likely due to the highly IL-10-producing monocytes (Figure 2B), overshadows the fact that monocytes from the HIV group are the highest producers of the a+b+6+8+10+T+ polyfunctionality (Figure 2A).

In vitro, the HIV group showed a significantly higher percentage of classical monocytes that produced intracellular IL-10 in response to LM-MS and LPS (Figure 3 and Supplementary Table 2). Similar results were observed analyzing the data as MFI (Supplementary Table 3). Multiple cytokine production was higher in the HIV group after stimulation with LM-MS, LPS, and ssRNA40 in the classical and intermediate monocytes, (Figure 4A). It is notable that the simultaneous composition of six cytokines was only observed in the HIV group. A more in-depth analysis of the 63 possible multiple cytokine functionality profiles in response to TLR agonist in vitro revealed that the cells producing IL-10 (10+) in combination with other cytokines was significantly higher in the HIV group. For instance, Figure 4B displays the multiple combinations of

six and five simultaneous cytokines in response to LPS in the classical monocytes among groups. Note that the HIV group displays the highest levels in all the multiple combinations that include IL-10, but not in the absence of IL-10. Additional information about single cytokine representation among polyfunctional distribution is shown in arcs in Supplementary Figure 2.

Association of soluble levels of inflammatory and coagulation biomarkers and the monocyte activation and functionality

We determined the soluble concentrations of inflammatory and coagulation biomarkers. The IL-1β and IL-10 levels were below the detection limit of the assay. We found that the IL-6 levels were significantly higher in the HIV group compared to the young control group with a trend for hsCRP and β2M (Supplementary Table 4). Interestingly, we found that in the unstimulated condition, the single cytokine production was positively and strongly correlated with soluble inflammatory levels in the three monocyte subsets when analyzing all study subjects as a whole group (n=60). Remarkably, the β2M, D-dimer and hsCRP levels were strongly and positively associated with the production of almost all of the pro-inflammatory intracellular cytokines, especially in the classical and intermediate subsets (Supplementary Figure 3). However, most of these associations were lost after stratification into groups (young, HIV and elderly), probably due to the small number of studied cases. Interestingly, the analysis of the HIV group separately revealed strong associations only significant for this subgroup. There was a strong inverse correlation between sCD14 levels and the IL-10 production (p=0.009, *rho*= -0.594) in the intermediate subset without stimulation and between the sCD163 levels and the IL-8 production in intermediate and non-classical subsets of monocytes also, in the unstimulated condition (p=0.003, *rho*= -0.629; p=0.016, *rho*= -0.531, respectively). When this analysis was applied to multiple cytokine combinations, in the three subsets, the polyfunctionality in all participants was also positively correlated with the soluble inflammatory markers in the unstimulated condition (data not shown).

Regarding the phenotype of activation markers, we found strong associations among activation cell surface markers on monocytes for the three groups of study. There was a strong positive correlation between CD11b and CD38 in the classical subset and a strong inverse association between CD49d and CD62L in the intermediate subset for each group (data not shown), strikingly the most significant and strongest associations between these markers were found for the HIV group (p<0.001, *rho*= 0.818; p<0.001,

rho= -0.68, respectively) Also, we have found associations between the activation phenotype and the soluble inflammatory concentrations. Of special interest were the associations of the scavenger receptor CD163 with the following in the elderly: sCD14 (*p*=0.045; *rho*=0.465), IL-6 (*p*=0.020; *rho*=0.542), hsCRP (*p*=0.029; *rho*=0.500) and β2M (*p*=0.004; *rho*=0.634). These associations were lost for all the plasma markers in the HIV group, with the exception of the sCD14 levels (*p*=0.028; *rho*=0.495).

DISCUSSION

Our results demonstrate profound phenotypic and functional alterations in the three monocyte subsets of HIV-infected patients on cART. Moreover, we showed that those alterations described in patients are different from the age-related changes. The results presented herein are the first to comprehensively characterize higher single and multiple intracellular cytokine production by monocytes both under unstimulated conditions and in response to TLR agonist in vitro in HIV patients when compared to uninfected, aged-matched and elderly subjects.

The results of the present study add novel evidence to the relevance of monocytes hyperactivation in the HIV infection scenario. Monocytes from immunologically well-conserved, young HIV-infected individuals on cART exhibited a higher expression of activation markers, which are implicated in activation, homing, migration and also stabilization of the adhesion of leukocytes to endothelial cells [9, 10, 14-16], even when compared to the elderly group (over 40 years older than the HIV group). These profound alterations may, therefore, promote the development of inflammatory diseases in HIV-infected patients by mechanisms different from those found in age-related illnesses.

Regarding polyfunctionality, this work adds a new concept to innate immunosenescence. In the T-cell scenario, immunosenescence is related to the changing phenotypic composition of T cells with age and impaired T-cell polyfunctionality [17]. In the HIV field, enhanced polyfunctionality in HIV-1-specific CD8+ T cells has been associated with better control of the virus [18]. Our results suggest that monocyte functionality in HIV-infected patients and controls is not desirable, because the production of intracellular cytokines in the unstimulated condition is positively correlated with soluble inflammatory and coagulation biomarkers that are known to be associated with increased morbidity and mortality risk.

Researchers have debated whether individuals with HIV infection experience accelerated aging (health risks associated with early aging), accentuated aging (health risks associated with aging are greater), or both. These speculations arise from the vast previous evidence from epidemiological studies [19, 20]. The critical question is whether HIV infection shares common mechanisms with the natural process of aging. Our results demonstrate that the concept of premature aging of monocytes and consequently the premature aging of the inflammatory systems of HIV-infected patients is a simplification of the mostly unknown and complex underlying alterations that HIV

infection produces in innate immune cells, which appear to be different from those of aging.

Monocytes are the main producer of IL-10 [21]; this cytokine is an important regulator of myeloid cells and has an anti-inflammatory role by down-regulating the capacity of monocytes to secrete inflammatory mediators, including IL-1α and TNF-α [22], providing a negative-feedback control loop. Here, we have observed an immunosuppressive mechanism that failed to inhibit proinflammatory regulation and homeostasis balance in HIV-infected patients on cART and that differs consistently from inflammatory homeostasis in aged subjects. The high levels of IL-10-producing monocytes found in the HIV group might be due to an unsuccessful attempt to reduce the high IL-6 plasma concentrations and the intracellular IL-6 levels observed in monocytes from these patients. One possible explanation of the high percentage of IL-10-producing monocytes that we have described as a specific alteration found in HIV patients, could be that the HIV trans-acting regulatory protein (Tat) directly induces IL-10 production by monocytes [23], interestingly through a CD14 independent mechanism [24]. A recent report has also described that Tat up-regulates PD-L1 expression on monocyte-derived dendritic cells (MoDCs) through TNF-α- and TLR4-mediated mechanisms, modulating the PD-1/PD-L1 T-cell coinhibitory pathway of antigen-presenting cells by hijacking the TLR4 pathway [25]. In the view of these results, the involvement of the Tat/TLR4/TNF-α pathway in the modulation of PD-1/PD-L1 and IL-10 production in monocytes in HIV-infected patients deserves further research.

Our study was limited by the small number of patients; however, the differences among the groups were clear. Other fact is that with the present study design we could not analyze the effect of cART on the activation of monocytes. It has been described in in vivo and in vitro studies of human cells and murine models that some nucleoside reverse-transcriptase inhibitors induced the activation of leukocytes [26, 27]. It will be interesting to analyze in further studies in what extent cART and the different types of antiretroviral treatments potentially affect the monocyte function. Finally, with this study design we could not analyze any cause and effect relationship to the monocyte activation; to achieve this objective longitudinal studies are now being conducted.

In summary, the chronic phenotype and inflammatory alterations associated with monocytes in HIV infection are maintained at a different level from those of aging. Our data points toward a crucial role of endogenously produced IL-10 in monocytes from

HIV-infected patients and provide evidence for an inflammatory homeostasis deregulation mechanism unable to constrain IL-6 production. The modulation of activated monocytes may have important clinical implications for mitigating the development of non-AIDS events (the main cause of morbidity and mortality in HIV-infected patients) and also for the design of therapeutic strategies in other chronic inflammatory conditions.

FIGURE LEGEND

Figure 1. HIV-infected patients exhibited a more activated phenotype of monocytes than the elderly. Percentage of surface expression of activation markers of monocyte subsets in HIV-infected patients on cART (HIV) and in two different HIV-uninfected controls: young (Y) and elderly (E) groups. The Mann–Whitney U test was used to compare the levels of activation markers between the HIV and different-age control groups. *p< .05 and ** p< .001. ns, not significant.

Figure 2. A) Percentage of classical monocytes producing six simultaneous cytokines (IL-1α, IL-1β, IL-6, IL-8, IL-10 and TNF-α) and single IL-6 and IL-10 production in the unstimulated condition between groups. * p< .05, ** p< .001 and ns, not significant. B) Polyfunctional cytokine distribution in the three subsets of monocytes under unstimulated conditions. Colors represent the simultaneous production of up to six cytokines (IL-1α, IL-1β, IL-6, IL-8, IL-10 and TNF-α) by monocytes among the groups (young (Y) and elderly (E)). The IL-10 production in polyfunctional distribution is shown in arcs. Statistically significant differences between the groups are shown as * p< .05,** p< .001 and ns, not significant. Pestle version 1.6.2 and Spice version 5.2 (M. Roederer. NIH. Bethesda. MD) were used.

Figure 3. The percentage of classical monocytes producing intracellular IL-10 in response to LM-MS and LPS between HIV-infected patients on cART (HIV) and different HIV-uninfected controls: young (Y), elderly (E) groups. The responsiveness of cells is represented as the difference between the stimulated and unstimulated values. The Mann–Whitney U test was used to compare the levels of activation markers between the HIV and different-age control groups. * p< .05, ** p< .001 and ns, not significant.

Figure 4. A) Polyfunctional cytokine distribution of the responsiveness in vitro to LM-MS, LPS and ssRNA40 in the three subsets of monocytes among the groups (young (Y), elderly (E) groups). Pie charts show classical, intermediate and non-classical monocytes with up to six functional responses. Statistically significant differences between the findings are shown. * p< .05, ** p< .001, and ns, not significant. p <0.1 is represented with Ω. B) The percentage of classical monocytes simultaneously producing multiple combinations of six and five cytokines (IL-1α, IL-1β, IL-6, IL-8, IL-10 and TNF-α) between groups in response to in vitro stimulation with LPS. Pestle version 1.6.2 and Spice version 5.2 (M. Roederer. NIH. Bethesda. MD) were used. * p< .05, ** p< .001 and ns, not significant.

Supplementary Figure 1. A) Schematic diagram of the gating strategy of a representative HIV-infected subject. Note that the conditions utilized minimize the shedding of CD16 and allowed the identification of the non-classical subset.B) Histogram representation of cytokine production on classical monocytes under unstimulated conditions (grey line) and after LPS stimulation (red line).

Supplementary Figure 2. Pie charts show the polyfunctional distribution of monocytes with up to six cytokines in response to LPS stimulation. Single cytokine representation among polyfunctional distribution is shown in arcs. Statistically significant differences between the findings are shown as * p< .05, ** p< .001 and ns, not significant. Pestle

version 1.6.2 and Spice version 5.2 software (M. Roederer. NIH. Bethesda. MD) were used.

Supplementary Figure 3. A) Associations between soluble inflammatory markers and the intracellular cytokine production in the three subsets of monocytes for all study subjects (n=60). Spearman's ρ correlation coefficient test was used. The *p* value is represented in italics and the *rho* value in bold. Statistical significance <0.05 is colored in black cells, *p* values <0.1 but >0.05 are colored in grey cells. B) Representative correlations of intracellular cytokine production and plasmatic concentrations in the three monocyte subsets for all study subjects (n=60).

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Table1. Characteristics of HIV–infected patients and healthy subjects

	Elderly (n=20)	**HIV (n=20)**	**Young (n=20)**	***p* (HIV *vs*. Elderly)**	***p* (HIV *vs.* Young)**
Sex (%Men)[+]	9 (45)	16 (80)	14 (70)	0.024	0.4
Age (years)[±]	83 [67-90]	37 [31-40]	35 [27-40]	<0.001	0.5
Absolute total monocyte count (cells/mm3)[±]	495 [393-623]	400 [300-498]	360 [310-400]	0.1	0.03
Anti-CMV-IgG titers (IU/mL)[±]	19.0 [9.9-27.3]	25.7 [16.1-38.0]	7.3 [0.1-18.2]	0.3	<0.001
CD4 T (cells/mm^3)[±]	666 [416-984]	733 [582-911]	753 [583-1040]	0.4	0.8
CD8 T (cells/mm^3)[±]	459 [304-811]	756 [630-1011]	467 [308-619]	0.009	<0.001
Ratio CD4:CD8 (cells/mm^3)[±]	1.4 [0.95-2.08]	1.0 [0.8-1.3]	1.7 [1.2-2.9]	0.049	<0.001

Values are presented as the percentage [+] or median and interquartile range [±]. Differences between groups were analyzed using the χ^2 test for categorical variables and the Mann–Whitney U-test for continuous variables.

Table 2. Percentage of unstimulated monocytes producing different intracellular cytokines among groups

	Unstimulated			***p value***	
	Elderly	**HIV**	Young	***p* (HIV *vs*. Elderly)**	***p* (HIV *vs*. Young)**
Classical monocytes (CD14++CD16-)					
IL-1α	0.4 [0.0-1.7]	0.1 [0.1-0.3]	0.0 [0.0-0.1]	0.2	0.003
IL-1β	0.4 [0.2-1.5]	0.3 [0.1-0.5]	0.1 [0.1-0.3]	0.3	0.017
IL-6	0.6 [0.3-1.2]	**1.8 [0.9-2.5]**	0.3 [0.1-0.5]	**<0.001**	**<0.001**
IL-8	2.3 [1.4-4.1]	1.5 [0.7-2.5]	1.2 [0.5-1.6]	0.1	0.2
IL-10	0.1 [0.0-0.5]	**7.5 [3.2-17.1]**	0.2 [0.0-0.8]	**<0.001**	**<0.001**
TNF-α	0.3 [0.1-0.4]	0.2 [0.1-0.5]	0.2 [0.1-0.3]	0.6	0.4
Intermediate monocytes (CD14++CD16+)					
IL-1α	1.9 [0.6-4.4]	0.3 [0.1-0.6]	0.2 [0.1-0.4]	<0.001	0.1
IL-1β	1.4 [0.6-3.9]	0.9 [0.4-1.8]	0.6 [0.2-0.8]	0.4	0.005
IL-6	1.3 [0.7-2.7]	**7.5 [4.5-10.1]**	0.6 [0.2-1.6]	**<0.001**	**<0.001**
IL-8	4.4 [1.9-7.6]	3.7 [2.3-7.9]	0.8 [0.4-3.9]	0.7	0.002
IL-10	2.1 [0.6-4.4]	**16.3 [8.6-25.3]**	0.4 [0.1-1.6]	**<0.001**	**<0.001**
TNF-α	0.6 [0.4-1.1]	0.6 [0.4-1.3]	0.3 [0.1-0.6]	0.9	0.011
Non-classical monocytes (CD14+CD16++)					
IL-1α	1.1 [0.6-2.1]	0.7 [0.3-1.8]	0.3 [0.2-0.6]	0.4	0.004
IL-1β	1.5 [0.8-3.9]	1.9 [0.7-4.2]	0.5 [0.2-1.2]	0.8	0.005
IL-6	0.8 [0.2-1.8]	**2.3 [0.9-5.2]**	0.2 [0.1-0.8]	**0.008**	**<0.001**
IL-8	6.8 [2.4-11.8]	4.5 [2.5-8.3]	1.5 [0.7-6.7]	0.5	0.018
IL-10	4.2 [1.1-9.9]	**15.8 [8.8-26.8]**	2.4 [1.1-5.2]	**<0.001**	**<0.001**
TNF-α	2.4 [1.1-3.9]	3.0 [1.4-4.9]	1.1 [0.5-1.6]	0.4	0.002

Values are expressed as the median and IQR. The Mann Whitney–U test was used to compare groups. Bold values indicate when HIV is significantly different from both control groups

Supplementary table 1. Median fluorescence intensity (MFI) of unstimulated monocytes producing different intracellular cytokines among groups

	Unstimulated			***p value***	
	Elderly	**HIV**	Young	***p* (HIV *vs*. Elderly)**	***p* (HIV *vs.***
Classical monocytes (CD14++CD16-)					
IL-1α	592 [582-610]	590 [486-633]	598 [572-678]	0.5	0.2
IL-1β	4037 [3586-4405]	4156 [3460-4900]	3583 [3025-4113]	0.4	0.02
IL-6	1108 [904-1578]	1332 [1304-1517]	1202 [913-1649]	0.05	0.3
IL-8	1833 [1569-2458]	**1588 [1399-1783]**	1825 [1648-2002]	**0.02**	**0.002**
IL-10	359 [250-500]	**608 [242-704]**	270 [190-369]	**0.03**	**0.003**
TNF-α	4197 [3078-6990]	3635 [3012-4113]	4165 [3237-5058]	0.3	0.1
Intermediate monocytes (CD14++CD16+)					
IL-1α	582 [445-605]	584 [520-658]	604 [446-685]	0.3	0.4
IL-1β	2485 [2312-3120]	2240 [2152-2314]	2268 [2030-3913]	0.001	0.4
IL-6	1309 [705-1501]	**1397 [1341-2188]**	943 [737-1970]	**0.05**	**0.008**
IL-8	1793 [1521-2697]	1676 [1114-2176]	1509 [1370-1569]	0.1	0.1
IL-10	350 [287-380]	**486 [292-645]**	307 [161-376]	**0.06**	**0.002**
TNF-α	3294 [2393-4218]	3960 [2640-5783]	2776 [2364-2982]	0.2	0.003
Non-classical monocytes (CD14+CD16++)					
IL-1α	576 [518-622]	573 [504-687]	560 [516-652]	0.7	0.9
IL-1β	2137 [1998-2524]	2051 [1968-2408]	2181 [1834-2719]	0.7	0.8
IL-6	1288 [712-1417]	**1844 [1626-2058]**	1239 [867-2490]	**<0.001**	**0.022**
IL-8	3296 [2237-4865]	3253 [2307-3459]	3193 [2565-4694]	0.3	0.4
IL-10	134 [107-159]	162 [136167]	139 [104-176]	0.01	0.1
TNF-α	1460 [1210-1574]	1117 [1067-1552]	1337 [1129-1478]	0.1	0.3

Values are expressed as the median and IQR. The Mann Whitney–U test was used to compare groups. Bold values indicate when HIV is significantly different from both control groups.

Supplementary Table 2. Percentage of monocytes producing different intracellular cytokines in response to TLR agonists among groups

	LMMS			LPS			ssRNA40			*p*					
	Elderly (1)	**HIV** (2)	Young (3)	Elderly (1)	**HIV** (2)	Young (3)	Elderly (1)	**HIV** (2)	Young (3)	**LMMS**		**LPS**		**ssRNA40**	
										1 vs 2	*2 vs* 3	*1 vs* 2	*2 vs* 3	*1 vs* 2	*2 vs* 3
Classical (CD14++CD16-)															
IL-1α	0.7 [0.1-3.4]	0.9 [0.3-3.6]	0.1 [0.0-0.5]	7.4 [0.4-21.3]	16.8 [1.8-23.7]	1.4 [0.6-6.6]	0.2 [0.0-0.8]	0.8 [0.4-1.2]	0.5 [0.2-1.3]	0.6	0.007	0.2	0.009	0.049	0.3
IL-1β	8.0 [3.1-15.5]	6.4 [2.1-10.0]	3.1 [1.3-5.8]	35.5 [18.9-	33.4 [7.1-70.2]	13.5 [4.3-34.9]	1.1 [0.5-1.7]	2.4 [1.4-.40]	1.2 [0.8-2.9]	0.3	0.1	0.9	0.1	0.017	0.2
IL-6	4.2 [2.5-9.0]	6.0 [3.7-12.7]	3.1 [1.6-6.7]	23.4 [10.5-	26.3 [10.0-	14.2 [4.5-34.5]	0.7 [0.0-1.2]	1.4 [0.5-3.0]	1.1 [0.6-2.1]	0.3	0.04	0.6	0.2	0.03	0.4
IL-8	15.3 [8.2-	11.4 [5.5-24.2]	12.6 [4.6-18.9]	44.2 [19.1-	34.6 [8.9-74.0]	26.2 [11.9-	1.7 [0.6-3.4]	1.4 [0.7-3.8]	1.9 [1.4.1-	0.3	0.6	0.6	0.4	0.9	0.4
IL-10	0.0 [0.0-0.2]	**3.5 [0.7-8.5]**	0.0 [0.0-08]	0.0 [0.0-1.3]	**4.0 [0.0-15.5]**	0.1 [0.0-0.8]	0.0 [0.0-0.1]	0.0 [0.0-8.5]	0.0 [0.0-0.1]	<0.001	<0.001	0.01	0.01	0.6	0.4
TNF-α	1.4 [0.6-2.7]	2.4 [1.2-6.1]	0.9 [0.4-1.9]	12.6 [4.1-25.5]	19.1 [3.3-50.8]	6.8 [1.7-15.0]	0.8 [0.2-1.3]	1.8 [0.8-2.4]	1.2 [0.6-2.4]	0.1	0.007	0.5	0.1	0.008	0.4
Intermediate															
IL-1α	2.5 [0.4-5.5]	1.0 [0.3-3.8]	0.2 [0.0-0.6]	15.5 [3.3-27.0]	12.1 [1.2-25.3]	1.7 [0.5-7.9]	0.6 [0.0-2.4]	0.7 [0.2-1.1]	0.3 [0.1-1.7]	0.2	0.006	0.9	0.1	0.9	0.6
IL-1β	9.3 [4.0-15.1]	9.8 [3.5-17.1]	2.9 [1.5-8.7]	32.7 [16.8-	25.9 [6.9-68.4]	10.6 [3.3-29.7]	0.8 [0.1-1.8]	2.4 [1.6-3.8]	1.4 [0.6-2.7]	0.9	0.01	0.8	0.2	0.01	0.1
IL-6	5.9 [3.6-11.4]	4.2 [0.9-9.2]	4.5 [1.9-7.8]	24.6 [8.8-40.9]	16.0 [4.2-46.3]	11.0 [4.8-21.6]	0.5 [0.0-1.2]	0.1 [0.0-1.7]	1.2 [0.3-2.4]	0.2	0.8	0.8	0.6	0.7	0.1
IL-8	14.2 [10.9-	14.6 [9.3-28.7]	10.8 [2.6-18.5]	36.4 [15.9-	26.8 [5.0-70.9]	21.4 [7.2-37.3]	1.1 [0.0-2.6]	1.8 [0.8-3.3]	1.7 [0.8-3.1]	0.9	0.1	0.7	0.6	0.3	0.3
IL-10	0.1 [0.0-1.7]	2.6 [0.0-8.1]	0.1 [0.0-0.8]	0.1 [0.0-1.6]	2.0 [0.0-15.1]	0.1 [0.0-1.6]	0.0 [0.0-0.7]	0.0 [0.0-9.2]	0.0 [0.0-0.5]	0.1	0.02	0.4	0.3	0.6	1
TNF-α	3.1 [1.3-5.5]	3.7 [2.5-7.7]	0.9 [0.5-2.7]	13.0 [2.8-29.2]	17.9 [3.3-52.0]	4.5 [1.9-14.0]	0.4 [0.1-1.3]	1.7 [1.1-2.2]	1.1 [0.5-1.6]	0.2	<0.001	0.5	0.1	0.004	0.1
Non-classical															
IL-1α	0.3 [0.0-2.0]	0.6 [0.3-1.3]	0.4 [0.0-1.1]	0.5 [0.0-0.9]	0.7 [0.2-1.0]	0.3 [0.0-1.6]	0.0 [0.0-0.3]	0.0 [0.0-0.2]	0.1 [0.0-0.3]	0.5	0.3	0.5	0.6	0.9	0.1
IL-1β	1.7 [0.5-4.9]	3.7 [0.8-6.0]	1.1 [0.5-3.4]	1.7 [0.0-5.0]	2.4 [1.0-7.8]	1.1 [0.2-1.9]	0.1 [0.0-1.1]	0.4 [0.0-1.7]	0.3 [0.0-0.9]	0.3	0.1	0.3	0.1	0.5	0.7
IL-6	0.8 [0.1-2.6]	0.0 [0.0-0.5]	0.3 [0.0-1.2]	0.9 [0.1-3.0]	0.0 [0.0-0.4]	0.2 [0.0-1.6]	0.0 [0.0-0.1]	0.1 [0.0-1.6]	0.1 [0.0-0.2]	0.004	0.02	0.002	0.02	0.5	0.8
IL-8	1.2 [0.0-4.2]	0.5 [0.0-4.0]	1.2 [0.4-4.3]	1.7 [0.2-6.4]	2.9 [0.1-5.1]	3.5 [0.3-6.4]	0.3 [0.0-2.6]	0.0 [0.0-1.3]	0.5 [0.0-2.4]	0.8	0.3	0.2	0.7	0.8	0.7
IL-10	0.8 [0.0-2.1]	0.0 [0.0-3.8]	0.4 [0.1-2.2]	0.0 [0.0-1.6]	0.0 [0.0-0.0]	0.6 [0.0-2.0]	0.0 [0.0-1.4]	0.0 [0.0-2.9]	0.0 [0.0-0.9]	0.8	0.4	0.8	0.02	0.8	0.9
TNF-α	1.7 [0.2-3.3]	1.1 [0.0-.4.2]	1.2 [0.3-4.78]	1.6 [0.2-3.6]	1.4 [0.1-3.4]	1.03 [0.2-4.0]	0.2 [0.0-0.8]	0.1 [0.0-2.1]	0.2 [0.0-0.8]	0.9	0.6	0.2	0.7	0.8	0.8

Values are expressed as Median and IQR. The Mann Whitney –U test was used to compare values of the elderly group to the young control group. Bold values indicate when HIV is significantly different from both control groups.

Supplementary Table 3. Median fluorescence intensity (MFI) of the intracellular cytokine production of monocytes in response to TLR agonists among groups

	LMMS			LPS			ssRNA40			*p*					
	Elderly (1)	**HIV (2)**	Young (3)	Elderly (1)	**HIV (2)**	Young (3)	Elderly (1)	**HIV (2)**	Young (3)	**LMMS**		**LPS**		**ssRNA40**	
										1 vs 2	*2 vs* 3	*1 vs* 2	*2 vs* 3	*1 vs* 2	*2 vs* 3
Classical (CD14++CD16-)															
IL-1α	13 [4-53]	32 [8-60]	1 [0.0-27]	50 [8-85]	**91[48-172]**	10 [0-26]	10 [0.0-36]	176 [46-225]	87 [0-172]	0.4	0.02	0.04	<0.001	<0.001	0.1
IL-1β	647 [430-1069]	433 [58-775]	51 [0-611]	2263 [1106-2794]	2210 [749-4326]	691 [17-1393]	1194 [346-	2590 [1452-	2586 [1076-4574]	0.04	0.1	0.8	0.06	0.07	0.8
IL-6	302 [215-357]	383 [192-441]	210 [17-407]	1213 [646-1868]	1472 [401-2714]	560 [331-1260]	219 [32-726]	247 [25-1139]	804 [150-1174]	0.1	0.03	0.4	0.7	0.6	0.9
IL-8	1083 [773-	1096 [776-1612]	839 [255-1205]	3347 [2407-4977]	3134 [1541-	1381 [997-3521]	383 [56-546]	1112 [378-1724]	800 [216-1703]	0.9	0.03	0.9	0.02	0.012	0.6
IL-10	2 [0-19]	**5 [3-19]**	1 [0-4]	0 [0.0-36]	**4 [0-10]**	0 [0-3]	0.0 [0.0-13]	0.0 [0.0-6]	6 [0-22]	0.06	0.001	0.02	0.02	0.7	0.09
TNF-α	718 [245-1343]	363 [67-660]	336 [6-826]	1207 [505-2324]	1258 [518-3419]	419 [0-1493	1841 [504-	5597 [4290-	3037 [1384-5882]	0.06	0.9	0.6	0.9	<0.001	0.1
Intermediate (CD14++CD16+)															
IL-1α	20 [3-55]	19[0-38]	18 [0-118]	69 [40-148]	98 [12-170]	11 [0-74]	7 [0-63]	100 [28-175]	66 [5-273]	0.6	0.7	0.8	0.03	0.05	0.8
IL-1β	668 [491-1428]	561 [382-986]	280 [13-614]	3262 [1694-3728]	1623 [628-5136]	872 [203-1601]	420 [179-1182]	719 [191-2338]	1160 [55-5045]	0.1	0.03	0.6	0.04	0.3	0.4
IL-6	438 [280-667]	170 [83-357]	165 [0-558]	1652 [862-2239]	713 [287-3501]	542 [48-1726]	86 [29-514]	36 [0-107]	294 [27-2993]	0.01	0.5	0.3	0.2	0.08	0.07
IL-8	693 [430-1068]	608 [328-1176]	334 [21-820]	2356 [980-4269]	1238 [613-6035]	1190 [353-1763]	112 [33-275]	159 [79-373]	536 [123-1847]	0.9	0.03	0.6	0.2	0.4	0.05
IL-10	0 [0-15]	3 [0-7]	2 [0-6]	0 [0-16]	6 [0-15]	4 [0-13]	0 [0-18]	2 [0-9]	1 [0-15]	0.6	0.9	0.6	0.8	0.7	0.7
TNF-α	1306 [929-	493 [46-965]	38 [0-410]	2540 [1382-5176]	1396 [592-4485]	445 [0-2414]	1368 [369-	1679 [1208-	2697 [447-10856]	0.03	0.6	0.2	0.1	0.2	0.6
Non-classical (CD14+CD16++)															
IL-1α	8 [0-27]	16 [0-68]	0 [0-3]	0 [0-33]	0 [0-32]	12 [0-30]	7 [0-14]	0.0 [0.0-62]	19 [0-75]	0.3	0.1	0.9	0.4	0.9	0.3
IL-1β	144 [0-312]	92 [0-182]	0 [0-283]	147 [0-360]	83 [0-273]	260 [0-400]	5 [0-201]	3 [0-30]	0 [0-479]	0.6	0.6	0.6	0.2	0.3	0.4
IL-6	42 [0-102]	14 [0-271]	159 [0-650]	119 [23-219]	61 [0-415]	57 [0-196]	29 [0-208]	0 [0-22]	5 [0-220]	0.8	0.3	0.8	0.6	0.05	0.2
IL-8	57 [0-187]	26 [0-68]	107 [0-493]	145 [0-343]	44 [0-146]	160 [74-366]	25 [0-136]	0 [0-47]	52 [0-400]	0.3	0.1	0.1	0.01	0.3	0.1
IL-10	3 [0-20]	3 [0-23]	6 [0-21]	8 [2-12]	2 [0-4]	3 [0-10]	0 [0-4]	2 [0-4]	1 [0-1]	0.8	0.4	0.05	0.2	0.8	0.6
TNF-α	72 [0-356]	84 [9-272]	166 [0-448]	97 [0-376]	145 [10-324]	74 [0-195]	0 [0-62]	17 [0-67]	100 [0-206]	0.9	0.7	0.7	0.3	0.4	0.3

Values are expressed as Median and IQR. The Mann Whitney –U test was used to compare values between HIV and control groups. Cells are colored when HIV is different from both control groups. Bold values indicate when HIV is significantly different from both control groups.

Supplementary table 4. Soluble levels of inflammatory and coagulation biomarkers.

Biomarker	**Elderly (n=20)**	**HIV (n=20)**	**Young (n=20)**	*p* (HIV *vs*. Elderly)	*p* (HIV *vs*.
IL-8 (pg/mL)	17.8 [10.1-36.4]	10.9 [7.1-15.2]	8.1 [6.9-18.5]	0.04	0.7
IL-6 (pg/mL)	3.3 [1.8-4.2]	1.7 [0.9-2.1]	0.7 [0.5-1.6]	0.01	0.03
TNF-α (pg/mL)	2.6 [1.9-3.3]	2.1 [1.1-2.9]	1.7 [1.1-2.4]	0.1	0.2
sCD14 (ng/mL)	8396.9 [7800.5-12089.6]	9014.8 [7157.0-11588.6]	7984.8 [6304.5-	0.7	0.4
hsCRP (mg/L)	2.6 [1.0-4.5]	1.1 [0.5-2.4]	0.6 [0.4-1.4]	0.1	0.1
β2-microglobulin	2.5 [2.1-3.3]	1.6 [1.5-1.8]	1.5 [1.4-1.7]	<0.001	0.1
D-dimer (µg/L)	820.0 [392.5-1647.5]	170.0 [170.0-465.5]	180.0 [170.0-250.0]	<0.001	1
sCD163 (ng/mL)	1423.2 [1123.1-1651.0]	931.4 [657.9-1124.2]	778.3 [561.9-1184.4]	<0.001	0.4

Median and IQR values are presented. The Mann–Whitney U test was used to compare the levels of inflammatory markers between group

Figure 1.

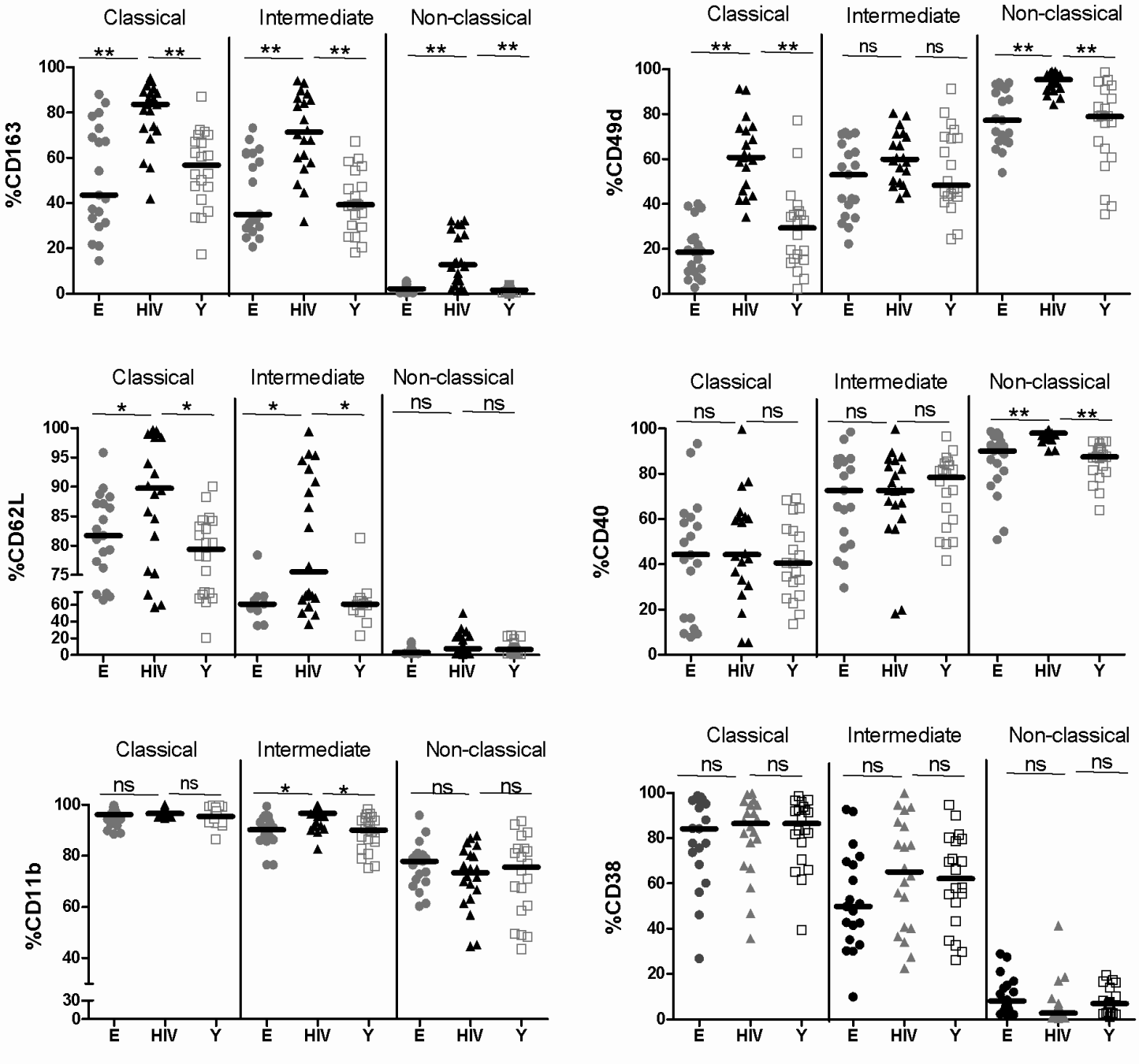

Classical
Intermediate
Non-classical
%CD163
%CD49d
%CD62L
%CD40
%CD11b
%CD38
E
HIV
Y
**
*
ns

Figure 2.

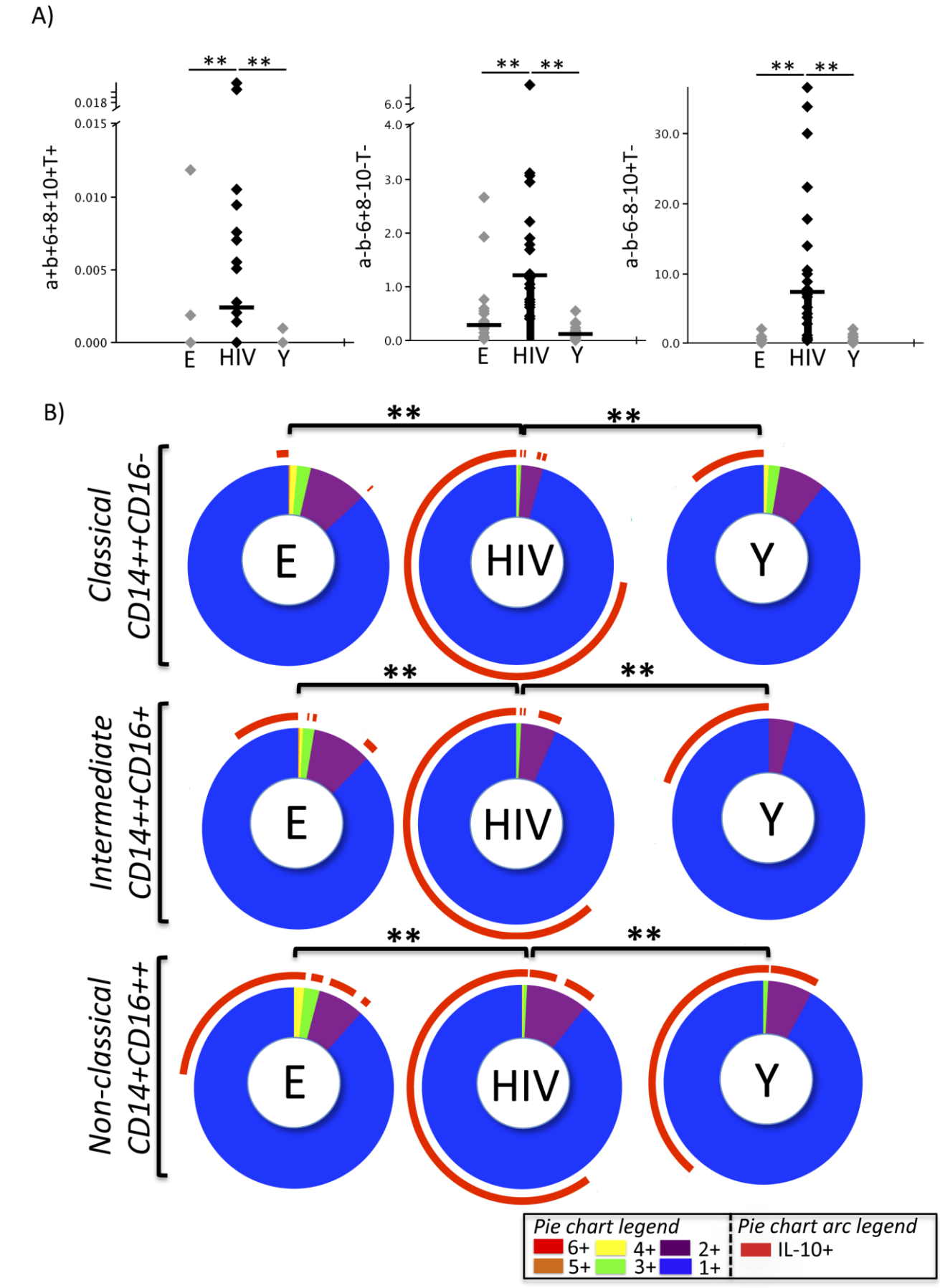

A)
a+b+6+8+10+T+
a-b-6+8-10-T-
a-b-6-8-10+T-
**
E
HIV
Y
B)
Classical CD14++CD16-
Intermediate CD14++CD16+
Non-classical CD14+CD16++
Pie chart legend
6+
4+
2+
5+
3+
1+
Pie chart arc legend
IL-10+

Figure 3.

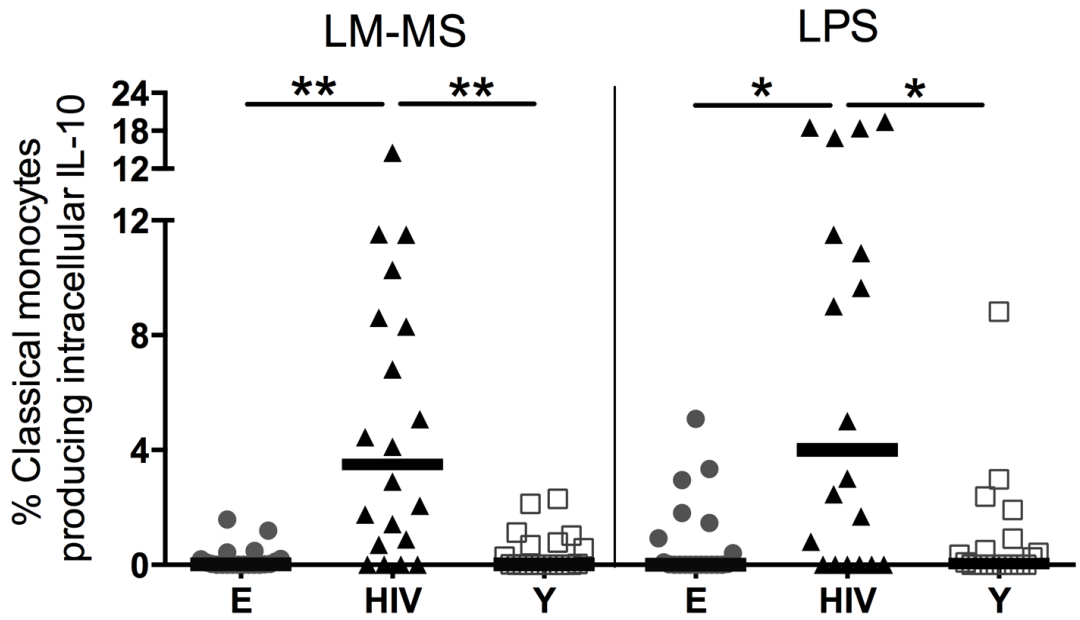
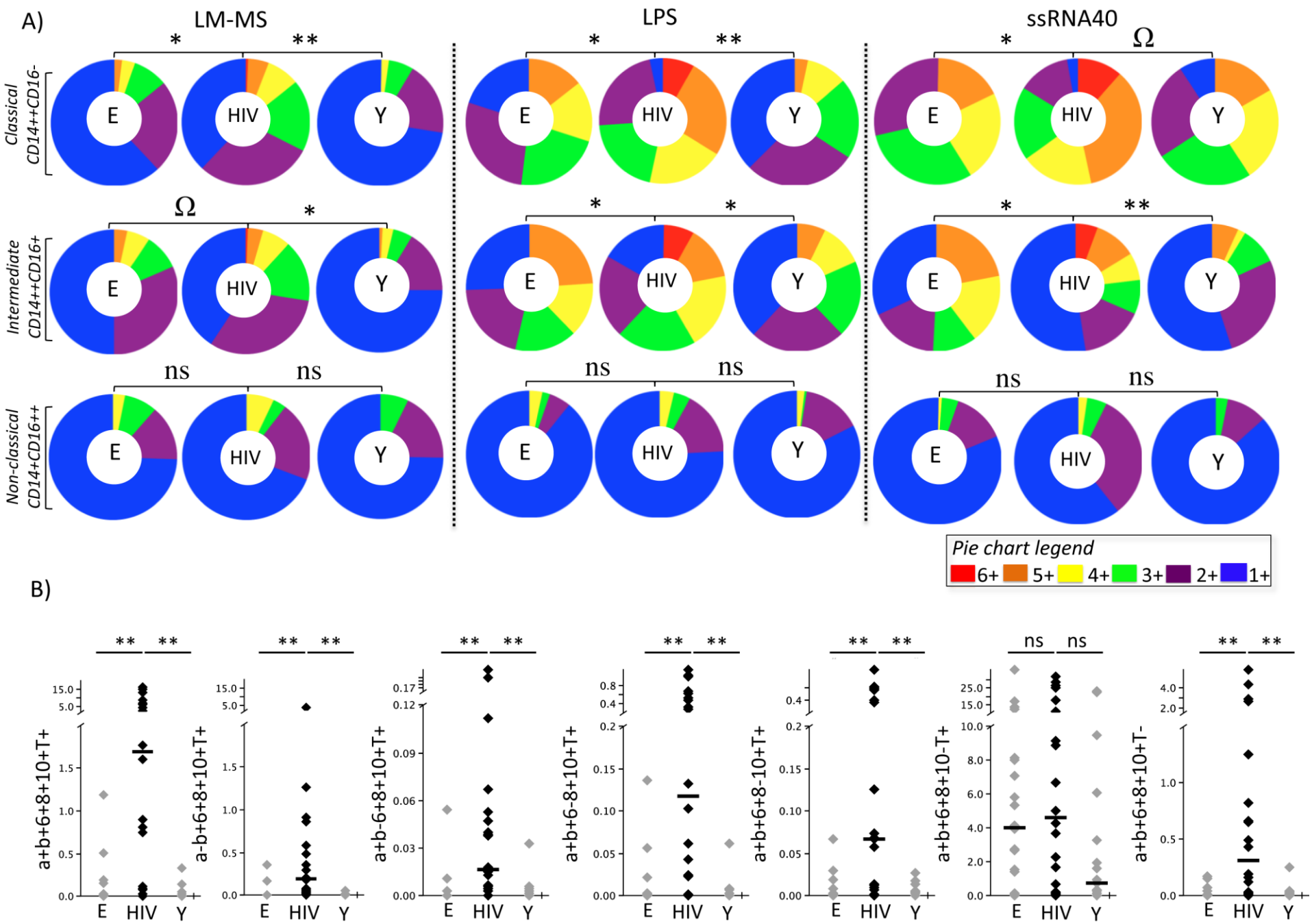
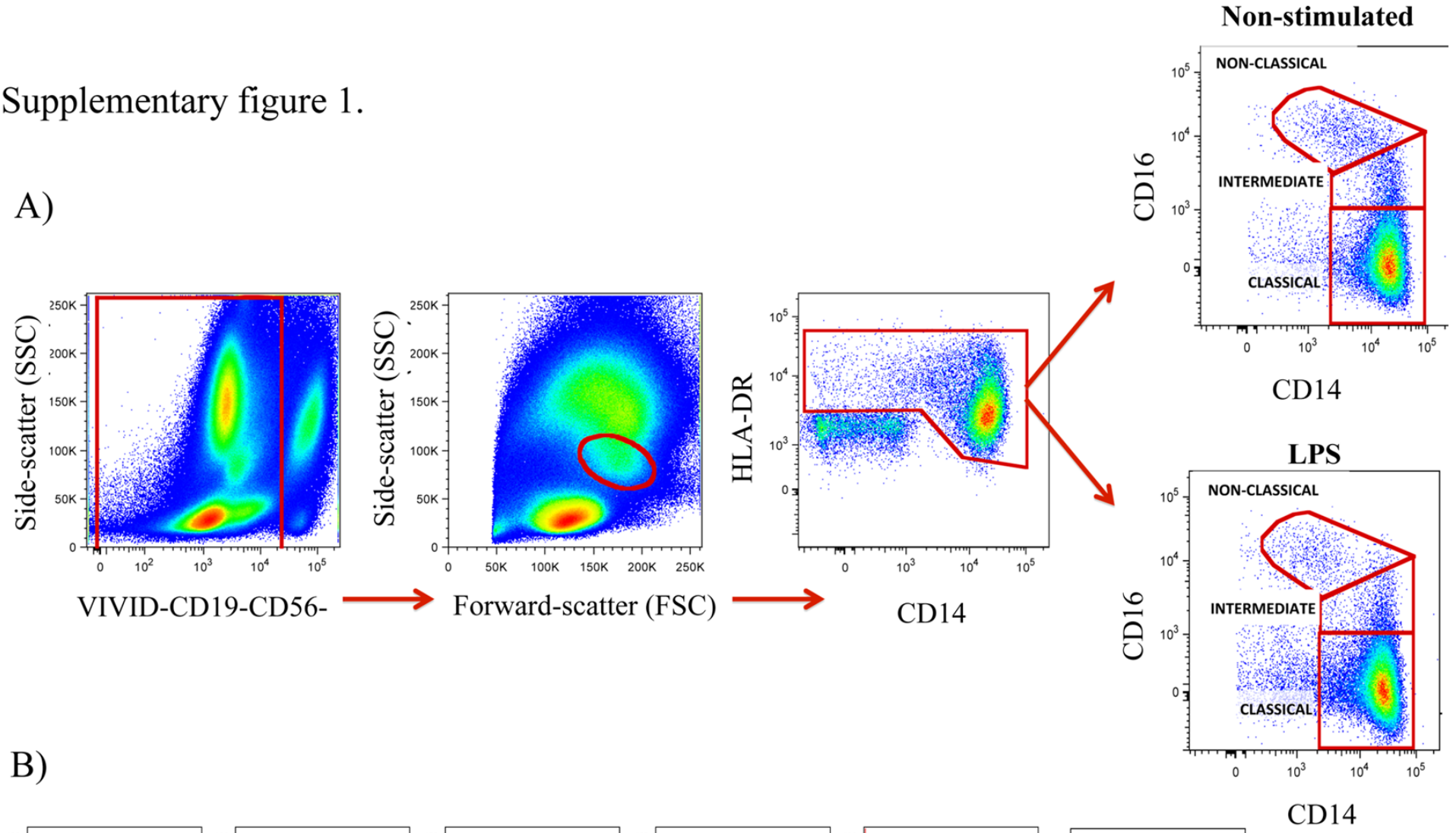

LM-MS
LPS
% Classical monocytes producing intracellular IL-10
24
18
12
12
8
4
0
**
**
*
*
E
HIV
Y
E
HIV
Y

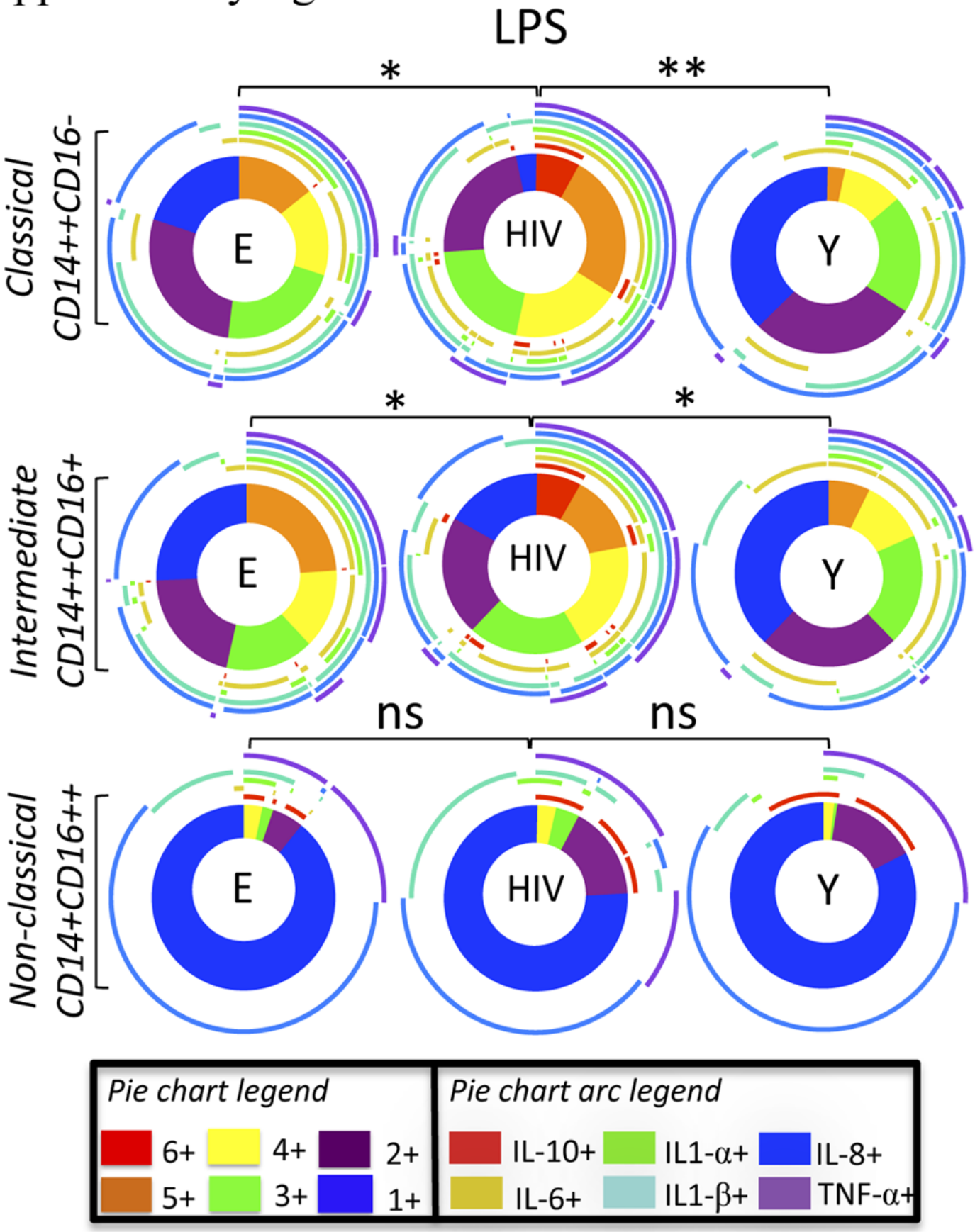
Figure 4.


A)
LM-MS
LPS
ssRNA40
Classical CD14++CD16-
Intermediate CD14++CD16+
Non-classical CD14+CD16++
E
HIV
Y
*
**
Ω
ns
Pie chart legend
6+
5+
4+
3+
2+
1+
B)
a+b+6+8+10+T+
a-b+6+8+10+T+
a+b-6+8+10+T+
a+b+6-8+10+T+
a+b+6+8-10+T+
a+b+6+8+10-T+
a+b+6+8+10+T-

Supplementary figure 1.



Supplementary figure 2.

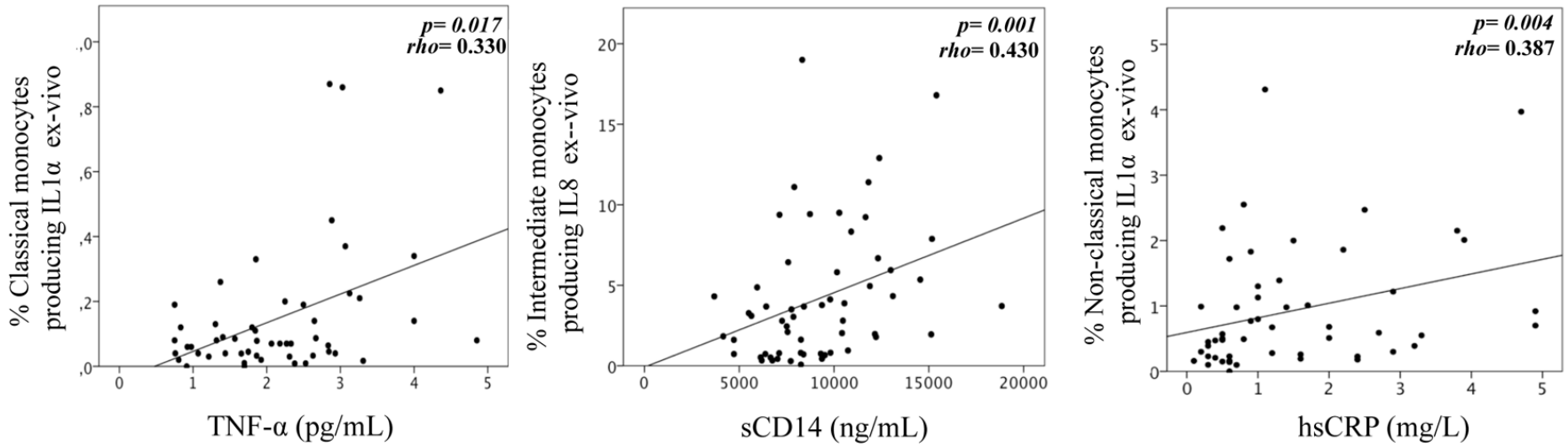


Supplementary figure 3.

A)

Soluble inflammatory and coagulation markers	% of monocyte subsets producing cytokines																	
	Classical						Intermediate						Non-classical					
	IL-1α	IL-1β	IL-6	IL-8	IL-10	TNFα	IL-1α	IL-1β	IL-6	IL-8	IL-10	TNFα	IL-1α	IL-1β	IL-6	IL-8	IL-10	TNFα
IL6 (pg/mL)	*0.037* / 0.294	*0.1* / 0.2	*0.4* / 0.1	*0.3* / 0.1	*0.8* / 0.0	*0.6* / 0.1	*0.1* / 0.2	*0.1* / 0.2	*0.5* / 0.1	*0.2* / 0.2	*0.7* / 0.0	*0.5* / 0.1	*0.016* / 0.309	*0.2* / 0.2	*0.8* / 0.0	*0.9* / 0.0	*0.6* / 0.0	*0.6* / 0.1
TNF-α (pg/mL)	*0.017* / 0.330	*0.6* / 0.1	*0.3* / 0.1	*0.6* / 0.1	*0.6* / 0.1	*0.2* / 0.1	*0.005* / 0.356	*0.9* / 0.0	*0.3* / 0.1	*0.2* / 0.2	*0.2* / 0.2	*0.2* / 0.2	*0.015* / 0.314	*0.6* / 0.1	*0.1* / 0.2	*0.7* / -0.1	*0.6* / -0.1	*0.9* / 0.0
IL8 (pg/mL)	*0.1* / 0.2	*0.8* / 0.0	*0.8* / 0.0	*0.4* / 0.1	*0.4* / -0.1	*0.7* / 0.0	*0.6* / 0.1	*0.8* / 0.0	*0.7* / 0.0	*0.9* / 0.0	*0.3* / -0.1	*0.2* / -0.2	*0.8* / 0.0	*0.6* / 0.1	*0.5* / 0.1	*0.3* / -0.2	*0.6* / 0.1	*0.4* / 0.1
sCD14 (ng/mL)	*0.1* / 0.2	*0.3* / 0.1	*0.2* / 0.2	*0.002* / 0.393	*0.4* / 0.1	*0.04* / 0.253	*0.8* / 0.0	*0.8* / 0.0	*0.3* / 0.1	*0.001* / 0.430	*0.6* / 0.1	*0.5* / 0.1	*0.8* / 0.0	*0.4* / 0.1	*0.4* / 0.1	*0.8* / 0.0	*0.4* / 0.1	*0.6* / 0.1
β2M (mcg/mL)	*0.049* / 0.263	*0.032* / 0.268	*0.1* / 0.2	*0.001* / 0.402	*0.1* / -0.2	*0.2* / 0.2	*0.002* / 0.394	*0.1* / 0.2	*0.8* / 0.0	*0.012* / 0.326	*0.6* / 0.0	*0.4* / 0.1	*0.1* / 0.2	*0.1* / 0.2	*0.5* / 0.1	*0.6* / 0.1	*0.7* / 0.0	*0.3* / 0.1
Ddimer (mcg/L)	*0.030* / 0.338	*0.014* / 0.345	*0.4* / 0.2	*0.009* / 0.374	*0.4* / -0.1	*0.4* / 0.2	*0.017* / 0.348	*0.048* / 0.376	*0.3* / 0.0	*0.1* / 0.2	*0.3* / 0.1	*0.6* / 0.1	*0.8* / 0.1	*0.024* / 0.355	*0.6* / 0.0	*0.3* / 0.2	*0.3* / 0.1	*0.04* / 0.253
hsCRP (mg/L)	*0.046* / 0.276	*0.1* / 0.2	*0.6* / 0.1	*0.4* / 0.1	*0.8* / 0.0	*0.4* / 0.1	*0.002* / 0.397	*0.005* / 0.359	*0.1* / 0.2	*0.2* / 0.2	*0.2* / 0.2	*0.1* / 0.2	*0.004* / 0.387	*0.1* / 0.3	*0.7* / 0.1	*0.5* / 0.1	*0.6* / 0.1	*0.6* / 0.1
sCD163(ng/L)	*0.1* / 0.2	*0.6* / 0.0	*0.4* / 0.0	*0.8* / 0.0	*0.4* / 0.0	*0.6* / 0.1	*0.1* / 0.2	*0.9* / 0.0	*0.3* / -0.1	*0.6* / 0.1	*0.4* / 0.1	*0.3* / 0.1	*0.006* / 0.353	*0.6* / 0.1	*0.3* / -0.1	*0.6* / 0.1	*0.1* / -0.2	*0.8* / 0.0

B)



Discusión

La presente tesis doctoral profundiza en el conocimiento del proceso de inmunosenescencia prematura en el contexto de la infección por VIH. Concretamente, aporta datos que sugieren que el paciente infectado por VIH-1 bajo cART no posee un envejecimiento prematuro ni de su sistema inflamatorio, ni de su sistema inmunitario innato, (centrándonos para ello en el estudio del monocito). Para ello, en primer lugar, describimos que los niveles de mediadores de inflamación solubles en pacientes infectados por VIH son diferentes a los observados en ancianos. En segundo lugar, caracterizamos la activación del monocito como posible mecanismo implicado en esa alteración del estado inflamatorio en ancianos y analizamos los posibles factores asociados en el envejecimiento natural; para con ello, en tercer lugar, poder compararlos con los observados en pacientes bajo cART y así concluir que los monocitos de este tipo de pacientes presentan una mayor polifuncionalidad y producción de IL-6 e IL-10 que no es compatible con un envejecimiento prematuro de estas células en el contexto de la infección por VIH.

En nuestras latitudes, en las que el acceso a la terapia antirretroviral combinada supresora de la CV es ampliamente extendida. Uno de los puntos más relevantes en relación a la patogénesis de la infección por VIH-1 es el manejo clínico del paciente bajo cART. Este tipo de pacientes, posee una creciente esperanza de vida (52), lo que conlleva que cada vez haya más pacientes que envejecen con la infección (5, 52, 53). En la actualidad, la mayoría de causas de muerte y enfermedad entre este tipo de pacientes son de origen inflamatorio (54-56). Por esto, profundizar en la caracterización de la alteración inflamatoria y en el estudio de los mecanismos asociados es una necesidad y un objetivo primordial por parte de la comunidad científica, que permitan desarrollar terapias de intervención en estos pacientes

En la presente tesis doctoral describimos que los niveles de marcadores inflamatorios en pacientes infectados por VIH eran diferentes a los observados no sólo cuando comparábamos una población control de similar edad, sino incluso al compararlos con una población envejecida. Así mismo, comunicamos que los niveles de TNF-α persistían elevados en esos pacientes a similares niveles a los observados en ancianos. Además, describimos que aquellos pacientes que poseían un mayor tiempo desde el diagnóstico de la infección, presentaban mayores niveles de IL-6, añadiendo más evidencia a la necesidad de diagnosticar y tratar a los pacientes en el menor tiempo posible (57-59). A la luz de esos resultados, discutimos en ese artículo que una de las causas que podrían estar asociadas a los altos niveles de marcadores proinflamatorios observados tanto en ancianos como en pacientes infectados por VIH, podría ser la elevada activación de monocitos circulantes, siendo éstos las células inmunitarias productoras de citoquinas proinflamatorias entre ellas, por excelencia, el TNF-α.

Los monocitos, son células heterogéneas. La caracterización de la funcionalidad de las tres subpoblaciones de monocitos en humanos es objetivo de estudios a nivel básico. Nosotros en la presente tesis doctoral, detallamos nuevos aspectos funcionales de las tres subpoblaciones con el

estudio de la polifuncionalidad de estas células y añadimos nuevos datos en análisis *ex-vivo* e *in-vitro* sobre la diferente respuesta ante agonistas de TLRs de las tres subpoblaciones de monocitos. En este sentido, describimos como los monocitos no clásicos presentaban la menor respuesta *in-vitro* ante agonistas de TLR2,4, siendo además esta subpoblación la mayor productora de TNF-α e IL-10 *ex-vivo*. Estos resultados confirman y añaden información sobre la funcionalidad de las poblaciones monocitarias. De forma interesante, observamos como la producción intracelular de citoquinas en las tres subpoblaciones (tanto de forma sencilla como la polifuncionalidad) se veía muy fuertemente asociada de forma directa con parámetros de inflamación y coagulación solubles, cuya implicación con el desarrollo de enfermedad y muerte ha sido ampliamente descrito en ancianos. El conjunto de estas observaciones, sugirieren fuertemente que el monocito es una célula clave promotora del estado de inflamación crónica y persistente de bajo grado descrito en ancianos, también denominado por otros autores como "*inflammaging"* (60,61).

La infección por CMV, es uno de los factores más consolidados como desencadenantes de los defectos y alteraciones observadas en inmunosenescencia, principalmente descritos en células T (62-64) y NK (65-68). El CMV, infecta células CD34+ y tiene un tropismo preferencial por monocitos, en los que establece infecciones latentes con reactivaciones intermitentes. Nosotros pensamos que la infección por CMV en ancianos podría ser un factor asociado a la activación de monocitos y a un deterioro en la función cognitiva de estos sujetos. Nuestros resultados demuestran una clara asociación entre la activación fenotípica de monocitos, los títulos de anticuerpos IgG frente a CMV (anti-CMV IgG) y el deterioro cognitivo medido por un test clínico como es el Minimental. Sin embargo, sólo podernos especular sobre la naturaleza de esas asociaciones, ya que el estudio no se diseñó para establecer relaciones de causa-efecto, por otra parte el significado biológico de los altos niveles de anti-CMV IgG todavía es objeto de estudio (69). Una cuestión que se deriva de estas observaciones es si los monocitos de estos pacientes están infectados por CMV y si la cuantificación del CMV intracelular en monocitos aislados de ancianos podría correlacionar de forma similar con la activación monocitaria y un bajo minimental test. Una posible explicación de las asociaciones observadas es la transmigración de monocitos infectados por CMV a sistema nervioso central mediante el paso de la barrera hematoencefálica (BHE) para rellenar el pool de microglía residente, cabría discutir dos puntos importantes que están siendo objetos de estudio en la actualidad como son por una parte la integridad de la BHE al paso de éstas células (70) y que la microgía (macrófagos residentes) puedan derivarse de monocitos circulantes (71,72). Otra de las teorías que podrían postularse, es que no fuera la infección de la línea monocito/macrófago por CMV directamente, sino la neuroinflamación asociada a un sistema inmune agotado que sufre mayores reactivaciones (73-75) la desencadenante del déficit cognitivo. Aproximaciones en este sentido, que podrían ayudar a comprender e interpretar los resultados serían la utilización de modelos animales *in-vivo*. Por ejemplo, evaluar si la infección piloto por CMV a ratones podría inducir cambios conductuales a ratones (por infectar el CMV directamente a su microglía) y diferenciarla del estado inflamatorio

sistémico y la neuroinflamación asociada, generado de forma alternativa por la inyección de LPS de forma crónica, en otro posible modelo *in-vivo* de ratón.

Tanto la infección por VIH como el envejecimiento natural se asocian con una serie de cambios en el sistema inmunitario de pacientes y ancianos, estas alteraciones especialmente han sido descritos en el compartimento celular T y se ha extendido por analogía al resto de componentes del sistema inmunitario. Una vez analizados los profundos cambios debidos a la edad en el envejecimiento natural, y puesto que describimos que el perfil de marcadores inflamatorios en pacientes y ancianos no es compatible con un simple proceso de aceleración del envejecimiento de su sistema inflamatorio. La siguiente cuestión que nos planteamos, caracterizar fenotípica y funcionalmente de forma detallada la activación monocitaria de pacientes infectados por VIH bajo cART supresor. Confirmamos y extendimos el concepto de que los monocitos de pacientes incluso bajo tratamiento antirretroviral a largo plazo están hiperactivados mediante el análisis de marcadores de membrana asociados (50). Encontramos altos niveles de expresión de marcadores como CD11b, CD49d, CD62L marcadores de activación implicados en la adhesión, rodamiento y transmigración de estas células; así como elevada expresión del receptor CD163, cuyos niveles solubles se han asociado con el desarrollo de aterosclerosis en pacientes, independientemente de otros factores de riesgo tradicionales (76). Esta elevada activación de marcadores implicados en la estabilización y adhesión de leucocitos a células endoteliales podría ser uno de los mecanismos implicados en el daño tisular y en último término en el desarrollo de enfermedades inflamatorias en pacientes (42, 48, 77). El análisis de la polifuncionalidad de las tres subpoblaciones de monocitos, de forma interesante nos mostró como los monocitos de pacientes infectados por VIH, presentan una mayor distribución de múltiples citoquinas, tanto en situaciones *ex-vivo* como *in-vitro*, en respuesta a agonistas de TLRs, especialmente en las subpoblaciones de monocitos clásicos e intermedios. Además, las concentraciones solubles de marcadores de coagulación e inflamación correlacionaban significativamente y de forma directa con las citoquinas intracelulares en la situación *ex-vivo*, lo que sugiere que en contra de lo que otros autores han comunicado para la polifuncionalidad de células T, en pacientes infectados por VIH se asocia con un mejor control del virus (78), la polifuncionalidad de monocitos no es beneficiosa, por asociarse con marcadores de inflamación y coagulación (ampliamente asociados a enfermedad y muerte) (12, 26, 79). En último lugar, en la presente tesis doctoral, aportamos evidencias que sostienen que el concepto de envejecimiento prematuro para definir las alteraciones observadas en pacientes infectados por VIH bajo cART, no refleja de forma objetiva las modificaciones observadas en la infección. Describimos una desregulación en los niveles de monocitos productores de IL-6 e IL-10 en pacientes, no compatible con la regulación de la homeostasis inflamatoria de jóvenes y ancianos. Los mecanismos implicados en esta elevada producción de IL-10 en pacientes, pensamos podría ser debido a un fallido intento de regulación negativa de los altos niveles de IL-6 observados en ancianos. El hecho, de observar este desbalance en la población VIH, también nos hace pensar que podría ser mediado por la propia infección, se ha descrito que la proteína específica de VIH tat (trans-acting regulatory protein) induce directamente la

producción de IL-10 en monocitos mediante un mecanismo CD14 independiente (80, 81). Por otra parte, nuestro estudio no se diseñó para evaluar los posibles efectos de diferentes tratamientos antirretrovirales sobre la activación de monocitos, en este sentido estudios *in-vitro* e *in*-vivo en modelos animales podrían ayudar a interpretar la implicación del tratamiento en los resultados generados. El conjunto de nuestros resultados sugiere que el término envejecimiento prematuro de monocitos es una simple generalización del conjunto de alteraciones de estas células y mecanismos implicados que en su mayor parte son todavía desconocidos. Es necesario seguir profundizando en el conocimiento de estas alteraciones ya que la modulación de la activación de estas células podría tener implicaciones clínicas que podrían disminuir el desarrollo de enfermedades inflamatorias en estos pacientes.

Conclusiones

1. Las alteraciones inflamatorias crónicas asociadas a la infección por VIH, se mantienen a diferente nivel a las observadas en el envejecimiento natural. Los elevados niveles de TNF-α, que persisten incluso tras el cART a largo plazo, cuando la replicación viral es controlada podrían causar daño tisular y tener implicaciones en el desarrollo de ENOS.

2. La producción intracelular de citoquinas ex-vivo en monocitos se asocia fuertemente con los niveles solubles de marcadores de inflamación y coagulación en ancianos, sugiriendo que los monocitos son uno de los principales mecanismos promotores del estado de inflamación crónica de bajo grado descrito en ancianos. La activación de monocitos se asocia con la infección por CMV y con el deterioro neurocognitivo de ancianos, sugiriendo que la infección por CMV podría ser uno de los mecanismos implicados en la activación de monocitos que como hemos observado se asocia con un déficit cognitivo en ancianos.

3. Las alteraciones inflamatorias relacionadas con monocitos en la infección por VIH son diferentes a las descritas en ancianos no infectados. La disfunción monocitaria, esencialmente, caracterizada por presentar en su mayoría mayores niveles de monocitos productores de IL-10 e IL-6, podría tener implicaciones en el desarrollo de ENOS, de forma diferente a las enfermedades asociadas al envejecimiento natural.

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